FERTILIZATION

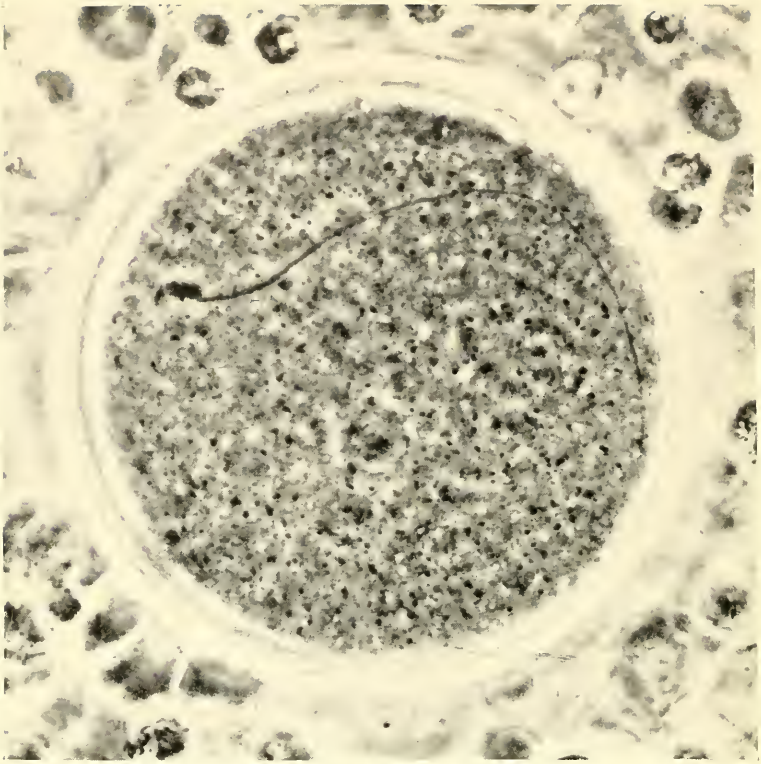


PLATE I

A live fertilized mouse egg, showing the whole spermatozoon in the cytoplasm. Positive phase contrast, × 770. Photograph by J. Smiles.

FERTILIZATION

BY

LORD ROTHSCHILD
G.M., Sc.D., F.R.S.

LONDON : METHUEN & CO. LTD
NEW YORK : JOHN WILEY & SONS, INC.

First published in 1956

CATALOGUE NO. 5807/U (METHUEN)

PRINTED AND BOUND IN GREAT BRITAIN BY
RICHARD CLAY AND COMPANY, LTD., BUNGAY, SUFFOLK

PREFACE

THIS book is intended for those who have read, or are reading, Gray's *Experimental Cytology*, Heilbrunn's *Outline of General Physiology*, Fruton & Simonds' *General Biochemistry*, Höber's *Physical Chemistry of Cells and Tissues* and similar textbooks. The subject is the life of the egg from the attachment of the fertilizing spermatozoon to the fusion or apposition of the male and female pronuclei. This process, except in mammalian eggs, usually takes a little less than one hour. Even so, several important subjects have had to be omitted: some of these are: (1) *Fertilization in the decapod crustacea and in sponges.* Both of these are too far removed from 'normal' fertilization to be included in a comparatively short book; but there are excellent accounts of them by Bloch (1935) and Tuzet (1950). (2) *Asters and the origin of the first cleavage amphiaster.* Much has been written recently on these, apart from the relevant sections in some of the textbooks mentioned above. (3) *Androgenesis and Gynogenesis.* (4) *Merogony.* (5) *Parthenogenesis.* A comprehensive review of parthenogenesis has been published by Tyler (1941*b*); but reference to the General Index will show that the subject is occasionally mentioned. (6) *Fertilization in the plant kingdom.* Although two chapters are devoted to this subject, its treatment is far from systematic.

The scope of this book precludes any discussion of cleavage, which is frustrating; not only because cell division is such a dominatingly important subject, but also because important papers such as Brachet's *Constitution anormale du noyau et metabolisme de l'embryon chez les Batraciens* (1954) cannot be considered.

References. Modern reviews sometimes consist mainly of a list of papers with little or no attempt at evaluation. Even if this practice served some useful purpose it would be inappropriate in a short book. The papers referred to represent a limited selection from the immense number on fertilization written during the last hundred years and, as a rule, I have excluded the following: (1) references to work which has recently been repeated, under more modern conditions. But attention is sometimes called to early papers on subjects in which there has been a revival of interest, such as cortical granules and the effect of calcium on the hardening

of the fertilization membrane, it having been forgotten or ignored that these were first described some forty years ago. (2) References to brief and scrappy papers which have not been followed up. Some exceptions to this rule will be found in chapter 7, Metabolic and Other Changes at Fertilization. (3) References to papers which I do not think good. Where work has been, or might be, wrongly accepted as true, I have drawn attention to the errors in it. But, in general, such papers have not been mentioned.

Every writer of a book on fertilization must be uncomfortably aware of his sins of omission and commission, so great is the labour imposed by the literature on the subject. The author is no exception and proffers his apologies.

Index of Plants and Animals. There are three columns in this index. The first gives the name of the organism, some of the familiar synonyms and the English or American names, when known. The second column states the order and class to which the animal or plant belongs. When I was a child, my father expected my sisters and myself to know the Latin names of the plants, bees and butterflies which we had to collect. It was inevitable therefore that an Index of Plants and Animals should figure in this book. But there was a more cogent reason. Reference will be found in several places to the specificity of fertilization, to the alleged specificity of the polysaccharides in egg jelly, and to interspecific, intergeneric and interphyletic cross-fertilization. We cannot think clearly about such subjects, nor describe and compare experiments relating to them, unless we are reasonably sure of the identity of the organisms concerned. Reference to the Echinoid synonyms shows that this is not always easy. A diverting example of the confusion which springs from careless nomenclature is to be found in a paper by Mitchison & Swann (1954*b*), which is discussed, for other reasons, in chapter 8. These authors measured the elastic modulus of the cortex of the unfertilized egg of the sea-urchin *Arbacia lixula* (Linn.). With the aid of their own and E. N. Harvey's measurements (1931), they calculated the tension at the surface of the unfertilized egg of an 'American species of *Arbacia*' (p. 469), which they refer to as *Arbacia pustulosa*. *Arbacia lixula* (Linn.) and *Arbacia pustulosa* (Leske) are synonyms for the same sea-urchin, although Harvey actually used the eggs of *Arbacia punctulata* (Lamarck) in the experiments in question.

Some Latin names of organisms are abbreviated after they have once been mentioned. If a reader is in doubt, reference to the Index of Plants and Animals will provide the full name. Its preparation was made very much easier by the assistance of Sir Gavin de Beer, F.R.S., and Dr H. W. Parker, of the Natural History Museum, London.

'Theories' of fertilization and activation. The desire to formulate a new theory of fertilization seems almost to be an occupational disease of the gametologist. Such theories have been connected, at one time or another, with the names of Boveri, Bataillon, Dalcq, Delage, Heilbrunn, F. R. Lillie and Loeb. At the present time we have gone too far—and yet not far enough—to formulate theories, or even to make an 'Attempt at a Comprehensive View', as Runnström tried to do in 1949. One function of a new theory is to provoke further experiments and, although I have no new theory of fertilization to offer, I hope that this end, at least, will be achieved.

Acknowledgments. I am particularly indebted to Professor Sir James Gray, F.R.S., who has been my mentor for twenty-five years; to Dr George Beadle, Chairman of the Biology Division of the California Institute of Technology, for his help and for his hospitality at 'Caltech', where most of this book was written; to Professor Albert Tyler for valuable advice and criticism, not all of which has been taken; and to the Medical Research Council for financial aid. In addition I am glad to record my thanks to the following scientists and non-scientists for their help and advice: Dr R. D. Allen; Dr C. R. Austin; Dr J. Beament; Miss G. Bending; Prof. J. Brachet; Miss M. Brewster; Dr R. R. A. Coombs; Prof. E. G. Cox, F.R.S.; Dr G. Fankhauser; Prof. L. V. Heilbrunn; Prof. A. L. Hodgkin, F.R.S.; Mr A. F. Huxley, F.R.S.; Dr G. W. Kenner; Dr M. E. Krahl; Dr T. R. R. Mann, F.R.S.; Dr R. Markham; Dr R. E. F. Matthews; Dr J. M. Mitchison; Dr F. Moewus; Prof. A. Monroy; Prof. C. Niemann; Prof. L. Pauling; the Hon. Miriam Rothschild; George Rylands; Prof. E. C. Slater; Prof. M. M. Swann; Dr E. Vasseur; and Prof. L. Zechmeister.

CONTENTS

PLATES

CHAPTER I

THE MORPHOLOGY OF FERTILIZATION

FERTILIZATION is the incitement of an egg to development by a spermatozoon, together with the transmission of male hereditary material to the egg. At fertilization the spermatozoon contributes *a*, the stimulus for development; *b*, a set of chromosomes embodying the paternal contribution to the genetic make-up of the zygote; and *c*, a central body which gives rise to, or is concerned with, the machinery for cell division. In some cases the spermatozoon, according to its point of entry into the egg, also determines the plane of bilateral symmetry of the embryo. Fertilization is specific and crosses between different sorts of animals are almost always impossible. Apart from a few exceptional cases to be discussed later, fertilization is irreversible. Once an egg has been fertilized, it cannot be re-fertilized, and once an egg has been stimulated to develop parthenogenetically, fertilization cannot be superimposed on parthenogenesis.

Fertilization can be divided into two phases. The first occurs when the homologous spermatozoon collides with and becomes attached to the egg surface. This is sufficient to set off a train of reactions in the egg which may lead to development. This first phase is called *activation* and one talks about a spermatozoon activating an egg or an egg being activated by a parthenogenetic agent. The spermatozoa of the worm *Rhabditis monohystera* Bütschli activate eggs of the same species so that they develop 'parthenogenetically', without containing any male hereditary material (Peacock, 1944). This phenomenon is known as pseudogamous fertilization and it can be achieved experimentally, by mixing homologous eggs and spermatozoa and separating them after a short time (F. R. Lillie, 1912*b*; Rothschild, 1953), or by heterologous insemination. Bataillon, for example, observed in 1909 that the spermatozoa of *Triturus alpestris* (Laurenti) activated the eggs of *Pelodytes punctatus* (Daudin) pseudogamously and it was this observation which led him to carry out his famous experiments on the parthenogenetic activation of frogs' eggs by puncturing them with fine glass needles. Both Loeb (1913) and

Godlewski (1912) made similar observations following heterologous insemination.

The second phase of fertilization is concerned with the events which take place after the spermatozoon has entered the egg, culminating in the disappearance of the sperm head and the egg nucleus as separate entities. Strictly speaking, therefore, fertilization begins with the sperm-egg collision and ends with syngamy, the fusion or apposition of the germ nuclei, when the spermatozoon loses its individuality. This series of reactions may take less than an hour; but the student of fertilization inevitably finds himself asking questions about the pre-fertilization behaviour of eggs and spermatozoa, the domain of the gametologist, and about the activity of the egg after syngamy, the domain of the embryologist. Examination of the pre-fertilization behaviour of the gametes must accompany any study of fertilization and this may well seduce the student away from his intractable problem. Mention has been made of spermatozoa colliding with eggs; why should they be anywhere near each other? Nature answers this question in bewildering and fantastic ways: the archegonia of plants produce chemicals which attract spermatozoa; dogfish and human beings rely on copulation to ensure sperm-egg collisions. Provided there is no moon, the male *Platynereis megalops* (Verrill) deposits spermatozoa in the mouth of the female, which bursts in about six seconds, liberating fertilized eggs into the sea (Just, 1914). Some further aspects of this problem, the liberation of spermatozoa and eggs in the right place and at the right time, are discussed in later chapters.

Maturation. The condition of the egg before fertilization, particularly as regards the stage of maturation it has reached, should always be borne in mind when trying to gain some understanding of fertilization. It has been insufficiently emphasized that echinoderm eggs, on which so many experiments have been carried out, are in an exceptional condition from the point of view of maturation, at the time of fertilization. Fig. 1, which is adapted from Dalcq (1952), explains this point. In sea-weeds, coelenterates, and echinoderms, and not all of them, the egg is fertilized after maturation (Class 4 fertilization). In all vertebrates and *Branchiostoma*, fertilization takes place at the second maturation metaphase (Class 3 fertilization), though there is some evidence that fox, dog and horse eggs may be fertilized in the germinal vesicle stage (Pearson & Enders, 1943; van der Stricht, 1923;

Hamilton & Day, 1945). In the eggs of *Ciona*, *Chaetopterus*, *Cumingia* and *Mytilus*, fertilization occurs at the first maturation metaphase (Class 2); while in sponges, *Nereis*, *Spisula*, *Urechis caupo* Fisher & MacGinitie (Plate II), *Ascaris* and *Sagitta*, the egg

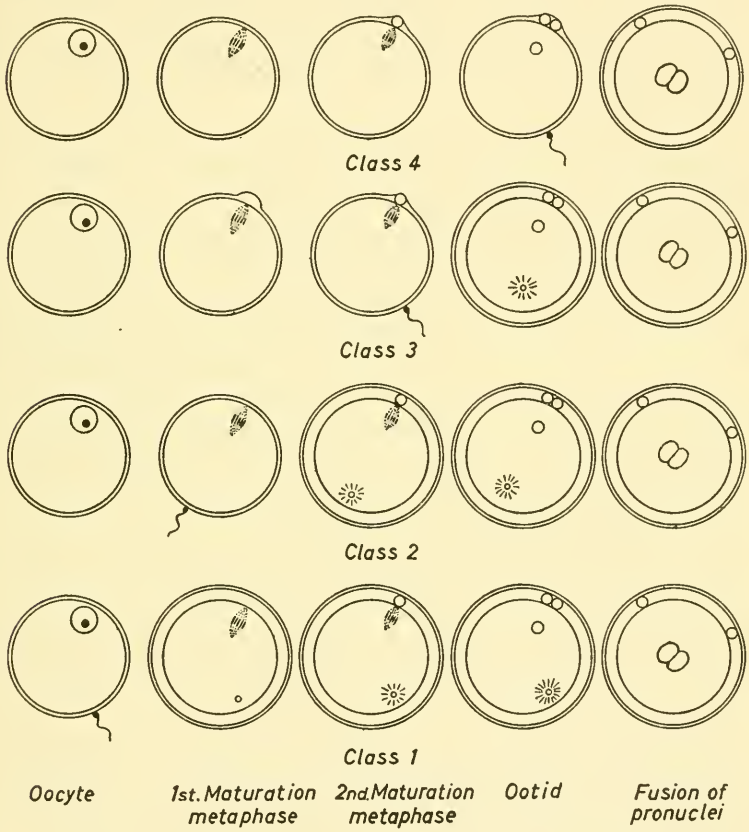


FIG. 1.—The four stages of egg maturation at which fertilization occurs in the animal kingdom, after Dalcq (1952).

is fertilized before the germinal vesicle of the oocyte has broken down, that is before either maturation division (Class 1).* These four classes should be remembered when studying fertilization, as we are sometimes tempted to make generalizations based on

* Needless to say, there are exceptions to this classification, e.g. starfish eggs, which come into Classes 1, 2, 3 and 4.

the behaviour of eggs in one class when those in other classes behave differently. There is, admittedly, a tendency for the sperm nucleus to remain relatively quiescent within the egg until after the formation of the second polar body; but this is not a sufficient reason for ignoring the fact that in the majority of *phyla*, fertilization does not occur at the same time in the life-history of the unfertilized egg as it does in sea-urchins. We shall return to this question when considering the metabolism of fertilized and unfertilized eggs.

Jelly and membranes. In some cases, there are what appear to be barriers between the egg and the spermatozoon. In echinoderms and frogs, for example, this barrier takes the form of a gelatinous shell round the unfertilized egg, through which the spermatozoon must bore or burrow to reach the egg surface. The egg of the salmon is surrounded by a rather tough chorion, which is impermeable to spermatozoa except at one point, the micropyle; this is a narrow channel in the chorion, through which spermatozoa must pass to reach the egg. When an unfertilized salmon egg is put into fresh water, the chorion hardens, the micropyle becomes occluded and the egg is unfertilizable. This is one of the reasons why breeders of trout and salmon mix eggs and spermatozoa 'dry', before dilution with fresh water, though not all of them realise that the success of 'dry' insemination is due to the egg micropyles remaining open in the presence of seminal plasma as opposed to fresh water. The other reason for mixing salmon or trout eggs with their respective semen in the 'dry' condition is because the spermatozoa of these fish only live for a few minutes after dilution with fresh water.

Many insect eggs are surrounded by hard and, one would have thought, impermeable egg shells, which contain several so-called micropyles. Insect spermatozoa, however, do not necessarily make use of these structures, which are often too small for the passage of a spermatozoon, and in many species, fertilization occurs before the egg shell is laid down. Insect spermatozoa are sometimes said to contain enzymes capable of dissolving or softening up egg shells. They can penetrate thin wax layers round the egg (Beament, 1946); but a careful perusal of Cragg's interesting paper (1920) on copulation in *Cimex lectularius* Linn. shows that the claim that bed bug spermatozoa can 'burrow' through chitin is less certain than has sometimes been thought. E. B. Wilson (1928) noticed that the

spermatozoa of *Cerebratulus* (*lacteus* Verrill?) pay no attention to the so-called micropyle present in the membrane round the eggs of this nemertine, and can reach and fertilize the egg at any point on the surface. The unfertilized egg of *Megathura crenulata* (Sowerby) is surrounded by a tough membrane which is distinct from the surface of the egg proper, and the same applies to human eggs, in which the enveloping membrane is called the *zona pellucida*. Spermatozoa get through these barriers with the help of enzymes located in their heads. Finally, most mammalian eggs are surrounded by follicle cells, in two layers; the innermost of these consists of densely packed, radially arranged cells and is known as the *corona radiata*. Outside this there is a layer of sparsely distributed cells, the *cumulus oophorus*. The enzyme hyaluronidase, contained in or on the surfaces of most mammalian spermatozoa, assists in the dissolution or depolymerisation of the intercellular cement, hyaluronic acid, by which the follicle cells are stuck to the unfertilized egg surface.

Cortical change. Having got through these 'barriers', the spermatozoon becomes attached to the surface of the unfertilized egg. After attachment, the sperm tail may continue to move quite vigorously, though in other cases, it sticks out from the egg surface, motionless. The first visible reaction of the egg to the attachment of the *fertilizing* spermatozoon—spermatozoa quite often become attached to eggs but fail to fertilize them—is a change in cortical structure, which, starting at the point of sperm attachment, passes completely over the egg surface. The time relationships of this reaction are discussed in chapter 9, Polyspermy. According to J. C. Dan (1950*a*, p. 402), this change in cortical structure is 'a visible wave which travels around the egg at speeds varying with the species, . . .'. 'In the relatively fluid eggs of *Mespilia* (*globulus* (Linn.)) the passage of this wave is especially striking; it causes a slight deformation of the surface layers of the egg, which gives the impression that some sort of tension is being progressively released, or that a local band of contraction and expansion is passing around the egg.' This wave of so-called contraction has been observed by numerous students of fertilization, but it is doubtful whether the word 'contraction' is apposite or even desirable, except in special cases such as that of the brook lamprey, *Entosphenus lamottenii* (Lesueur), which does contract after fertilization (Okkelberg, 1914), or in the case of mammalian eggs. The German word

Schrumpfung (wrinkling), roughening, granulation, or simply cortical change are nearer the facts. Moser (1939*a*) examined this reaction in the eggs of *Arbacia punctulata*. He found that a layer of cortical granules immediately below the plasma membrane, diameter 0·8 μ, disappeared at fertilization, the disappearance starting at the point of attachment of the fertilizing spermatozoon and passing progressively over the egg surface, in about 10 seconds at 26° C. A breakdown of cortical granules in the eggs of *Sabellaria vulgaris* Verrill, 5–10 minutes after fertilization, was described in the same year by Novikoff (1939). Moser's studies were followed up by Endo (1952), who observed that at fertilization, the cortical granules, of which there are about 0·6/μ^2 in the eggs of *Clypeaster japonicus* Döderlein, doubled their diameters and then exploded. Just before they disappear, sea-urchin egg cortical granules, which, according to Monné & Hårde (1951), contain polysaccharides esterified with sulphuric acid residues, exhibit Brownian movement, which suggests that at this time, the cortex becomes more fluid (Allen, 1954). A similar phenomenon occurs when fish eggs and those of the marine worm *Nereis succinea* (Leuckart) are fertilized, though in these, alveoli in the cortex break down progressively after fertilization (Yamamoto, 1944; Kusa, 1953; F. R. Lillie, 1919). In addition, Kusa (1954) has shown that the cortical alveoli in the egg of the dog salmon, *Oncorhynchus keta* (Walbaum), contain mucopolysaccharides esterified with sulphuric acid residues. As regards the cortical response to fertilization, there is, therefore, a marked chemical and morphological resemblance between fish and echinoderm eggs. But, as we shall see later, it would at present be dangerous to ascribe too important or dominating a role to exploding cortical granules or discharging cortical alveoli in fertilization.

There has been some misunderstanding (Allen, 1954), perhaps of a verbal nature, about the disappearance of the cortical granules and the change in the light-scattering properties of the egg surface at fertilization, when viewed with dark-ground illumination. There is no doubt that the cortical granules disappear, but at the same time, the cortex becomes more *granular*, or roughened. Rothschild & Swann (1949) suggested that this granulation, which is associated with an increase in light scattering, might be due to the formation of microscopic or sub-microscopic particles at the egg surface. The appearance of this granulation naturally does not imply that

the cortical granules remain unchanged after fertilization. Both phenomena occur and are intimately related to each other. The disappearing cortical granules are concerned in the formation of a structure which appears round some eggs after fertilization, the Fertilization Membrane (q.v.).

Fertilization cone. After attachment of the spermatozoon, a conical hyaline protuberance, the fertilization or entrance cone, appears at the egg surface, Fig. 2. In the eggs of *Psammechinus miliaris* (P. L. S. Müller), the fertilization cone disappears in less

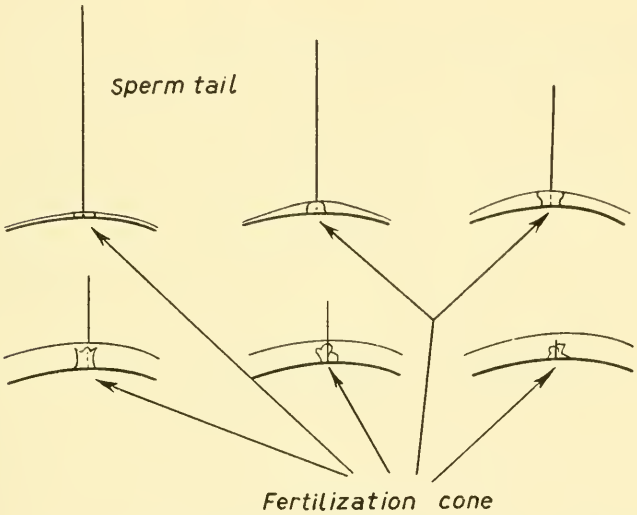


FIG. 2.—Entry of the spermatozoon into the egg of *Patiria pectinifer* (J. Müller & Troschel), after J. C. Dan (1950*a*).

than 20 seconds at 18° C., but in other eggs it may persist for much longer. In the case of the egg shown in Fig. 2, for example, the fertilization cone is visible until the tail of the spermatozoon has passed into the egg cytoplasm, after which it is more or less resorbed into the egg.

Sperm-egg filaments. In 1877 Fol reported that the starfish spermatozoon became connected to the surface of the egg by a long, exceedingly thin filament, which he believed was an extension of the fertilization cone. This observation was confirmed by R. Chambers in 1923, contradicted by Just in 1929 and reaffirmed by Hörstadius in 1939. Similar claims, that filaments derived from

the egg pull the spermatozoon towards the egg surface, have been made elsewhere; for example, Colwin & Colwin (1949) reported that a thread-like structure connected the fertilizing spermatozoon to the fertilization cone in the egg of *Saccoglossus kowalewskyi* (A. Agassiz), while Monroy (1948) refers to the fertilizing spermatozoon of *Pomatoceros triqueter* (Linn.) being connected to the egg surface by a thread. In the case of the starfish spermatozoon, J. C. Dan (1954) has shown that in certain circumstances, a thin filament, about 25 μ long and 0·13 μ in diameter, can be observed protruding from the front end of the head. Although immature eggs respond to insemination, and therefore to sperm-egg collisions, by emitting filament-like structures (E. B. Harvey, 1938), Dan's work leaves little doubt that Fol, R. Chambers and Hörstadius were wrong in thinking that the starfish egg responds to a nearby spermatozoon by emitting a filament which joins the egg to the spermatozoon and pulls the latter towards the egg surface. The presence of long filaments on the front ends of sperm heads may be of wider incidence than has hitherto been realised. Rothschild & Tyler (1955), for example, have reported their occurrence in the spermatozoa of *Echinocardium cordatum* (Pennant), *Mytilus edulis* (Linn.), *Strongylocentrotus purpuratus* (Stimpson) and *Lepidochitona cinerea* (Linn.). There are, however, some spermatozoa, e.g. those of the bull and ram, in which such filaments do not exist. The subject of acrosomal filaments and their role in fertilization is still very much in its infancy. In a recent paper, J. C. Dan (1955) has adduced convincing evidence that the spermatozoa of Japanese sea-urchins eject acrosomal filaments in the presence of sea water in which eggs of the same species have been standing, though the reaction does not occur if the calcium content of the medium is reduced. Do some spermatozoa always have acrosomal filaments on their heads and others only after responding to some stimulus? Further experiments are needed to resolve this interesting and important question, which has been brought into prominence mainly through the work of J. C. Dan.

Fertilization membrane. Unfertilized echinoderm eggs are surrounded by a vitelline membrane outside the plasma membrane, Fig. 3. At fertilization and shortly after the cortical change, the vitelline membrane separates from the egg surface, the separation starting at the point of sperm attachment and passing progressively over the egg surface (Kacser, 1955). After this, the vitelline mem-

brane becomes known as the fertilization membrane, which is about 500 Å thick (Mitchison, 1953). As will be seen from an examination of Fig. 3, the cortical granules which disappear at

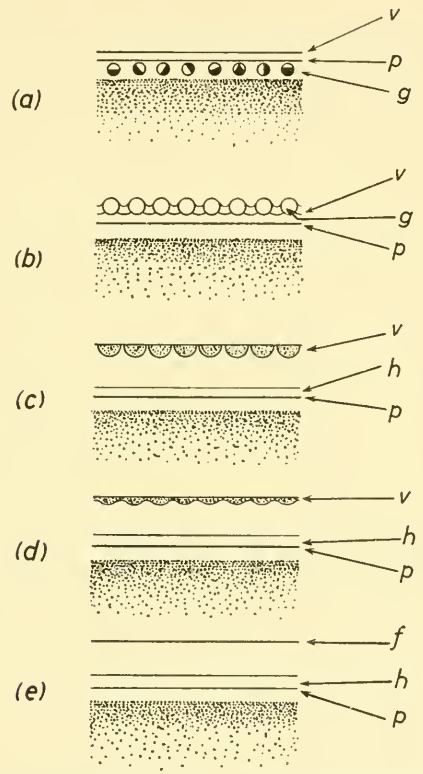


FIG. 3.—Formation of the fertilization membrane in the sea-urchin egg, after Endo (1952). *a*, Unfertilized egg; *b*, extrusion of cortical granules; *c*, adhesion of cortical granules to vitelline membrane; *d*, further transformation of fertilization membrane; *e*, completely transformed fertilization membrane. *v*, vitelline membrane; *p*, plasma membrane; *g*, cortical granules; *h*, hyaline layer; *f*, fertilization membrane. *Note.* Diffraction effects at the surface of a large egg make it extremely difficult to distinguish by optical methods closely apposed layers which are less than 1–2 μ thick.

fertilization in fact fuse with the inner surface of the vitelline membrane, a phenomenon which was first systematically examined by Motomura (1936, 1941), though Just observed the escape of granules from the cortex, their appearance in the perivitelline space, and possibly their incorporation into the fertilization

membrane, as early as 1919.* Endo (1952) has published some remarkable photographs of cortical granules adhering separately to the inner surface of the fertilization membrane of *Clypeaster* eggs. The space between the fertilization membrane and the surface of the egg is called the perivitelline space, an unfortunate term as the vitelline membrane is outside this space, not inside it. Globular isotropic cortical granules can sometimes be seen in the perivitelline space, where they may undergo a spontaneous transformation into positively birefringent rod-shaped particles. If unfertilized eggs are treated with trypsin and then fertilized, these rod-shaped particles are clearly visible. The fusion of the transformed cortical granules with the vitelline membrane is responsible for its hardening and transformation into the fertilization membrane, which takes place during the first ten minutes after fertilization. Calcium ions and a third factor which can be extracted from eggs are also concerned in the hardening or 'tanning' of the fertilization membrane (Motomura, 1950, 1954; Runnström, 1951). The properties of the fertilization membrane have been studied in great detail under a variety of environmental conditions by Runnström and his colleagues. A detailed review of this subject will be found in *The Cell Surface in Relation to Fertilization* by Runnström (1952). One interesting property of the fertilization membrane, which has not received sufficient mention, was described in some detail by Pasteels in 1950. He observed that the fertilization membranes of the eggs of *Chaetopterus variopedatus* (Renier), *Nereis succinea*, and of *Spisula solidissima* (Dillwyn), are contractile. More accurately, at certain times after fertilization the membrane 'expands', thereby becoming creased or folded. The effect is soon reversed and the membrane re-assumes its usual smooth (contracted?) and spherical appearance. The region on the fertilization membrane where this folding and unfolding phenomenon first occurs, transiently, 20 minutes after fertilization in *Chaetopterus* eggs, is at the vegetative pole, i.e. 180° away from the point of expulsion of the first polar body. The same happens after the expulsion of the second polar body, 30 minutes after fertilization, while 5 minutes later, the

* Cortical granules and their behaviour at fertilization are much in the limelight at present; it is therefore only right to mention that some forty-five years ago, E. N. Harvey (1911, p. 523), said that in the eggs of *Arbacia punctulata*, there were 'numerous minute stained granules, quite unmoved by the centrifuge. At the time of fertilization these disappear, apparently going to form the substance which passes out of the egg and hardens to a fertilization membrane'! The stain used was neutral red.

fertilization membrane is smooth and spherical again. The phenomenon is observed again just before the first division. There is little doubt that these changes in the structure of the fertilization membrane are caused or triggered off by the diffusion of certain substances from the cortex into the perivitelline space. It is an open question whether the fertilization membrane has the power of self-propagation once it has been activated, or whether the propagation of the effect is due to the progressive diffusion of a substance out of the cortex, the escaping substance simply causing a localised expansion of the membrane.

The mechanism responsible for the elevation, as opposed to the formation, of the fertilization membrane is not clear, though it has always been assumed that release of substances between the vitelline and plasma membranes, to which the former is impermeable, causes an influx of water with consequent formation of the perivitelline space. The escaping (and exploding?) cortical granules could conceivably be responsible for this allegedly osmotic phenomenon. Apart from these considerations, the contents of the perivitelline space, in which the concentration of solids is about 0·07% (Mitchison & Swann, 1953), require further investigation, as in spite of the observations of Gray (1927) and R. Chambers (1942), which suggest that the perivitelline fluid is liquid, Hiramoto (1954) has recently claimed, as Fol did in 1879, that it has viscous-elastic properties which can be removed by treatment with calcium-free sea water.

Structures somewhat similar to the fertilization membranes of echinoderm eggs are found in other eggs, for example in those of *Branchiostoma*, prototherian mammals, *Ascaris*, the frog and trout. In the frog's egg the membrane in question is called the vitelline membrane and in that of the trout, the chorion. In both cases the membrane separates or peels off from the egg surface, after fertilization; but the same thing happens, though much more slowly, in the frog's egg, if the unfertilized egg is left in tap water, from which one might conclude that these eggs sustain an abortive parthenogenetic stimulus by immersion in fresh water. Białaszewicz (1912) produced rather convincing evidence that the growth of the perivitelline space in frogs' eggs was an osmotic phenomenon associated with the presence of proteins, derived from the egg, in the perivitelline space.

There is no phenomenon comparable to the elevation of the

fertilization membrane in mammalian eggs; these eggs are, however, surrounded by a membrane, the *zona pellucida*, whose structure changes shortly after fertilization (Smithberg, 1953; Braden *et al.*, 1954), in a way which is somewhat reminiscent of the 'tanning' of the fertilization membrane. As a result of this change, the *zona pellucida* becomes less permeable to spermatozoa. Braden *et al.* (1954) believe that the change in the permeability of the *zona pellucida* to supernumerary spermatozoa is a reaction propagated within the *zona*; but their experiments do not entirely rule out an alternative hypothesis, that a substance progressively released from the egg surface into the perivitelline space is responsible for the 'tanning' of the *zona* (see chapter 9).

Entrance of sperm tail and middle-piece. In *Anthocidaris crassispina* (A. Agassiz) the sperm tail lies motionless outside the fertilization membrane for about $2\frac{1}{2}$ minutes after fertilization. It then straightens out, radially to the egg surface, and starts moving vigorously. According to J. C. Dan (1950*a*), this movement makes the sperm tail enter the egg in 15–30 seconds. There seems to be no fixed rule as to whether the tail and middle-piece of a spermatozoon enter the egg at fertilization. In *Nereis succinea*, neither the tail nor the middle-piece go in with the head (F. R. Lillie, 1912*b*), while in *Nyctalus noctula* (Schreber), the whole spermatozoon is found in the cytoplasm (van der Stricht, 1902). In spite of the classical and painstaking research of Meves (1912) on the fate of the middle-piece in those cases where it does enter the egg, and in spite of the fact that the whole spermatozoon often (or perhaps always) enters the mammalian egg (Blandau & Odor, 1952; Austin, 1953; Shettles, 1954), the existing evidence does not permit the conclusion that either the middle-piece or the tail of the spermatozoon has an important function in fertilization, after attachment of the fertilizing spermatozoon. This is to be expected in the case of the sperm tail, which is obviously an organ of locomotion, at any rate in animal spermatozoa.* Recent studies suggest that the middle-piece may contain mitochondria-like material concerned with locomotion, and endogenous substrates (Rothschild & Cleland, 1952). Austin would probably not agree with these views, as he has recently said (1953, p. 196), in regard to fertilization

* It may be that some of the tails of plant spermatozoa have a sensory function, apart from being locomotor organs; but there is no evidence to support this idea at present.

in mammals, that 'It seems clear that, in all the species investigated, the whole sperm enters the egg and that the cytoplasmic components of the mid-piece mingle with the egg cytoplasm and thus contribute something to the embryo.' Before any progress can be made in resolving this question, we shall have to try and find out what this 'something' is, and what its importance is to the embryo.

To turn from the echinoderms and examine the early phases of fertilization in a different *phylum* is both interesting and instructive. In the unfertilized egg of *Spirocodon saltatrix* (Tilesius), the egg nucleus lies at the base of a slight depression on the egg surface, this depression appearing after the extrusion of the second polar body. J. C. Dan says (1950*b*) that the spermatozoon invariably enters the egg in the immediate neighbourhood of the female nucleus; also that other spermatozoa accumulate round this part of the egg. These observations raise the possibility that sperm chemotaxis, for which there is very little reliable evidence in the animal kingdom, may occur in the coelenterates. Immediately after the sperm head has penetrated into the cortex, a tubular structure develops round its tail. The growth and degeneration of this structure are shown in Fig. 4, at various times after the beginning of fertilization. There are no membranes round the egg, before or after fertilization. The sperm tail passes completely into the egg cytoplasm in about 15 minutes and during this time it is in continual but slight movement.

The earliest phases of fertilization have now been observed in mammalian eggs; Shettles (1954), for example, has reported the successful fertilization of human eggs *in vitro*. Earlier claims of success in this field have been sympathetically but firmly reviewed by Austin (1951*b*) and Smith (1951).

Hyaline Layer. Soon after the elevation of the fertilization membrane, the so-called hyaline layer or Hyaloplasm appears on the surface of sea-urchin eggs. This is a thin, extracellular and gelatinous layer which dissolves in calcium-free sea water. As this layer can be removed without affecting the viability of sea-urchin eggs for a considerable time, it used to be thought that this structure was an extraneous membrane whose main function was to hold the blastomeres together after cleavage. The suggestion has, however, recently been made that the hyaline layer may play some part in preventing more than one spermatozoon entering an

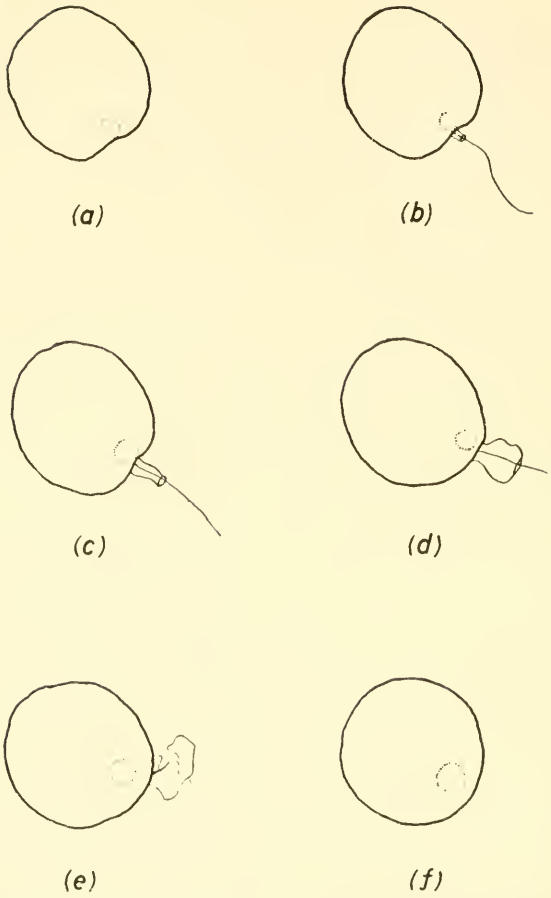


FIG. 4.—Entry of spermatozoon into the egg of *Spirocodon saltatrix*, after J. C. Dan (1950*b*). *a*, Unfertilized egg showing female nucleus at periphery of egg and depression in egg surface; *b*, 30 sec. after fertilization (*f*.); *c*, 3 min. 35 sec. after *f*. Development of 'fertilization tube'; *d*, 7 min. 15 sec. after *f*. Swelling and incipient disintegration of fertilization tube; *e*, 11 min. 20 sec. after *f*. Further disintegration of fertilization tube and only tip of sperm tail visible outside egg; *f*, 15 min. 20 sec. after *f*. Spermatozoon completely engulfed, fertilization tube disintegrated and swollen fusion nucleus moving away from the periphery towards centre of egg.

egg (Hagström & Hagström, 1954*c*; Hagström & Allen, 1956). We shall examine this idea more closely in a later chapter, specifically concerned with polyspermy.

Movements of the male and female pronuclei. After passing through the cortex, the head of the spermatozoon rotates through

180 degrees so that the anterior end points outwards, in the direction from which the spermatozoon entered. According to E. B. Wilson (1928, p. 423) the rotation of the sperm head is 'a very widespread if not universal phenomenon'. This is an exaggeration as it definitely does *not* occur in rat or mouse eggs. Rotation of the sperm head occurs rather quickly, in 2–3 minutes, in the egg of *Lytechinus variegatus* (Lamarck). Nothing is known about the mechanism underlying the phenomenon.

The subsequent movements of the male and female pronuclei present the following problem: in the unfertilized egg the female pronucleus may be situated almost anywhere in the cytoplasm; it may be in the centre of the egg, or at the periphery, as in Fig. 4. Usually, the spermatozoon may enter the egg at any point on the surface and after passing through the cortex, becomes the male pronucleus with its surrounding aster, a prominent structure in many, but not all, eggs, formed in the cytoplasm under the influence of the sperm head. Ultimately, the male and female pronuclei meet at about the centre of the egg. How do they get there? The first stage in trying to answer this question is to examine the morphology of the reaction. One of the most detailed descriptions of the movements of the pronuclei in normal, uncompressed eggs is that of E. L. Chambers (1939). Chambers says that, in spherical eggs, the male pronucleus moves at a uniform speed* towards the centre of the egg, along a straight line at right angles to the egg surface, Fig. 5. In the older literature, summarised by E. B. Wilson (1928) and also by Chambers, the male pronucleus was said to travel to the centre of the egg along a curved path which could be resolved into two components, a *penetration path* at right angles to the egg surface; and a *copulation path*, towards the female pronucleus. Chambers thinks, probably rightly, that this curved path is abnormal, at any rate in Class 4 fertilization, and due to the compression of the egg, or to the egg being non-spherical. It seems likely that the movement of the male pronucleus to the centre of the egg is caused by the growth of the sperm aster round it. If so, when the rays of the growing sperm aster come up against the inside of the egg surface, their elongation simply pushes the male pronucleus towards the centre of the egg. According to E. B. Wilson (1902), this explanation is

* According to Allen (1954), the velocity of the male pronucleus is far from uniform; but his experiments were done on eggs which had been sucked into narrow capillaries and which were therefore deformed in shape.

invalidated by an experiment in which the development of the sperm aster was inhibited by treating eggs with ether shortly after fertilization. He claimed that in these circumstances the movements of the male pronucleus were unaffected. Wilson makes no reference to this experiment in his famous textbook *The Cell in Development and Heredity*, and the ether experiment should be

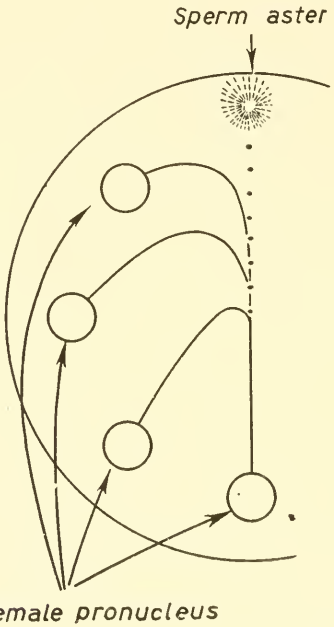


FIG. 5.—Path of male pronucleus from periphery of egg, labelled sperm aster, towards centre of egg, the path being shown by · · · ·. Four alternative paths of female pronucleus to centre of egg, according to original position in the unfertilized egg, are also shown. After E. L. Chambers (1939).

treated with reserve unless it is repeated. At the same time, Conklin's examination (1905) of fertilization in *Styela partita* (Stimpson) shows that in some cases, at any rate, protoplasmic streaming may affect the movements of the male pronucleus. As a general rule, however, and until experiments of the Chambers type are done on eggs other than those of echinoderms, and particularly on mammalian eggs in which there is nothing comparable to the sea-urchin or frog egg sperm aster, the movement of the male pronucleus must be assumed to be a straightforward mechanical phenomenon, caused by the growth of the sperm aster (only, of

course, in those cases where a sperm aster exists). In di- and tri-spermic eggs the paths of the male pronuclei are clearly curved; but there is no need to postulate the existence of fancy forces as an explanation. When the pronuclei get near enough to each other, they are pushed apart by their own growing asters.

The movements of the female pronucleus are not so straightforward. Fig. 5 shows the various paths followed by the female pronucleus, according to its original position in the unfertilized egg. If these movements are connected with the sperm aster, the influence of the latter must extend far beyond its visible boundaries. While the female pronucleus is moving through the cytoplasm, granules can be seen moving with it and at the same rate; but once it reaches and starts moving through the sperm aster, always radially with respect to the astral rays, no synchronous granule movements can be observed. The velocity is markedly non-uniform, which is understandable, first because of the inertia of the female pronucleus, which will affect its initial movements, and secondly, because of the gel-like consistency of the sperm aster through which it travels to the male pronucleus. There can be no doubt that the male pronucleus 'attracts' the female pronucleus. This remark is nothing more than the verbal equivalent of what one can see in Fig. 5. If the male pronucleus were not present, the path of the female pronucleus would be different. But there is no implication that the male pronucleus attracts the female pronucleus by electrostatic or electromagnetic forces; nor that long range forces, which were almost as popular as hyaluronidase a few years ago, have anything to do with the phenomenon. The male pronucleus might, purely for example, be responsible for cytoplasmic currents which move the female pronucleus in the required direction. Chambers made the interesting observation that if the female pronucleus happened to be in the centre of the egg, it moved *away* from this position to enter the sperm aster. He believes that in the initial stages of its movement, the female pronucleus is subjected to an attractive stimulus towards the centre of the sperm aster; the direction of this attractive stimulus continually changes as the sperm aster moves towards the middle of the egg. When close to the male pronucleus, Chambers suggests that a further stimulus ('component of force', p. 418) acts on the female pronucleus so that, although approaching the male pronucleus, it also travels parallel with it. This second component,

which is responsible for the two pronuclei taking up a central position within the egg, is what used to be called the *Cleavage Path* of the fusion nucleus. The movements of the female pronucleus towards the male pronucleus are thought by Chambers to be caused by cytoplasmic streaming towards the middle of the male pronucleus.

This description of the movements of the pronuclei may seem complicated, but in fact it is over-simplified in several respects; for example, little or no attention has been paid to the somewhat conflicting results obtained by earlier workers such as Fol (1879) or to the path of the male pronucleus in Classes 1–3 fertilization. A more serious difficulty concerns the behaviour of the female pronucleus in the absence of a male pronucleus. As is well known, activation makes the egg nucleus swell. But, as Moore showed in 1937, the female pronucleus not only swells after parthenogenetic activation but also moves to the centre of the egg under its own steam. The same occurs after pseudogamous fertilization. Rothschild (1953) reproduced a photograph of several pseudogamous sea-urchin eggs, in which a swollen female pronucleus can be clearly seen in the centre of the egg, though in this species, *Paracentrotus lividus* (Lamarck), the nucleus of the unfertilized egg is often eccentrically placed in the cytoplasm.

To sum up, the movement of the male pronucleus may be caused by the growth of the sperm aster, at any rate in Class 4 eggs; the movement of the female pronucleus is influenced by, but not entirely dependent on, the male pronucleus. Further experiments on the morphology and mechanics of the reaction are clearly needed before much will be learnt from chemical and biochemical studies.

Austin (1951*a*) has given an excellent account of the formation, growth and conjugation of the pronuclei in the rat egg. The most striking features of the process on the male side are the 'dissolution' of the sperm head 10–60 minutes after entering the egg, and the appearance of numerous male nucleoli which swell and ultimately coalesce to form the male pronucleus. On the female side, fertilization catalyses the completion of maturation and the development of female nucleoli which also swell and coalesce, to form the female pronucleus. As mentioned earlier, asters are far less prominent than in the eggs of the frog or sea-urchin.

Changes in the shape and volume of the egg. One of the most consistent features of fertilization is that eggs change their shape at

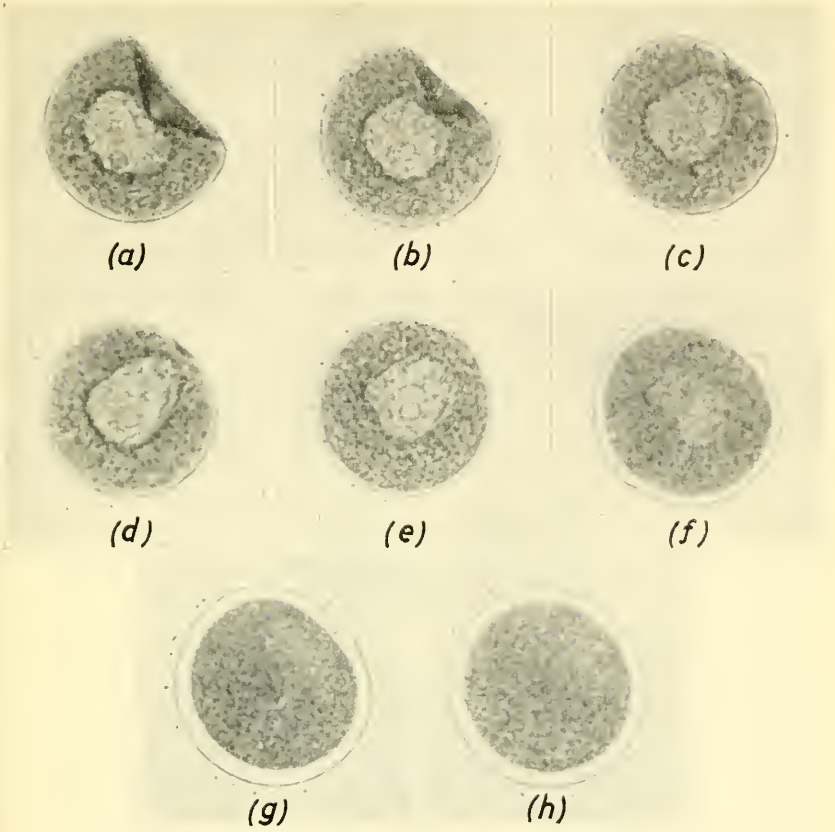


PLATE II

Fertilization of the egg of *Urechis caupo*. *a*, unfertilized, with intact germinal vesicle and invagination; *b–h*, 1, 2, 3, 5, 12, 30 and 35 min. after fertilization. Note changes in shape and polar body extrusion. Tyler (1932).

fertilization, such changes always resulting in the egg becoming more spherical. For example, the unfertilized egg of *Urechis caupo* has a large indentation in its surface, which disappears a few minutes after fertilization, the egg becoming spherical, Plate II

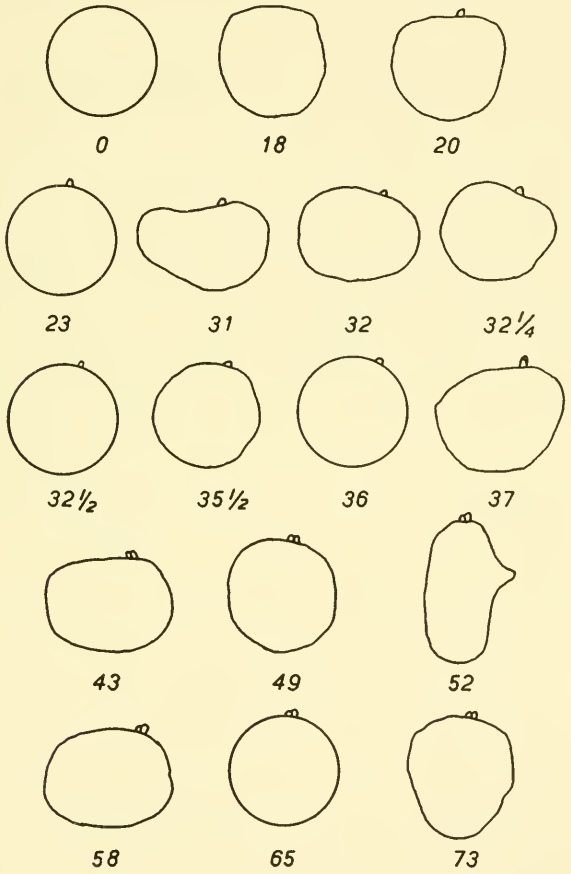


FIG. 6.—Changes in the shape of the egg of *Ascidiella aspersa* (O. F. Müller) after fertilization (external membranes removed), after Cohen & Berrill (1936). The numbers below each drawing are minutes after fertilization.

(Tyler, 1932). The eggs of *Cumingia tellinoides* (Conrad) are not spherical before fertilization, become transiently ovoid thirty seconds after fertilization and round up in about a minute (Morgan & Tyler, 1930), while every student knows that some batches of sea-urchin eggs are pear-shaped before, and completely spherical

a few minutes after, fertilization. The same occurs when the eggs of *Saccoglossus kowalewskyi* and *Thalassema neptuni* Gaertner are fertilized (Colwin & Colwin, 1953; Hobson, 1928). Changes in the shape of the eggs of *Ascidiella aspersa* after fertilization are shown in Fig. 6. Rounding up after fertilization is not due to the influx of water, but to changes in the physical properties of the cortex. These are discussed in a later chapter.

According to Glaser (1913, 1914, 1924), there is an 8% reduction in the volume of *Arbacia* eggs at fertilization, but this has been denied by R. Chambers (1921). An 8% change in volume would not be easy to establish with any certainty by measurement of egg diameters; but I do not believe that any reduction occurs in the eggs of *Echinus esculentus* Linn. or *Psammechinus miliaris*. A reduction in volume definitely occurs at fertilization in the eggs of the brook lamprey, of *Ascaris equorum* Goeze (Fauré-Frémiet, 1913), of *Hydroides norvegicus* Gunnerus (Monroy, 1954), of *Chaetopterus variopedatus* (Monroy, 1954), and of a number of mammals (Pincus, 1936).

CHAPTER 2

SPERM-EGG INTERACTING SUBSTANCES, I

THE spermatozoa and eggs of many animals and plants contain or produce substances which have well-defined effects on the gametes of the opposite sex and, in some instances, on those of the same sex. These substances are sometimes known as Gamones (= gamete hormones), though this word is not in general use. They have also been given other names: F. R. Lillie (1912*a*) published the first systematic account of one of these substances, derived from unfertilized eggs, which he called *Fertilizin*. In certain circumstances a solution of fertilizin agglutinates spermatozoa of the same species. The substance on the head of the spermatozoon with which fertilizin reacts is known as *Antifertilizin*; it was first extracted from spermatozoa by Frank (1939) and Tyler (1939), while later, Tyler (1940*a*) also extracted it from eggs. The biological and chemical characteristics of sperm-egg interacting substances are summarized in Table 1, which also contains a list of their various names.

Reciprocal induction of spawning. Among aquatic organisms it has been known for many years that a spawning female often induces males and other females of the same species to shed their gametes, and *vice versa*. For example, spawning females of the acorn worm, *Saccoglossus horsti* Brambell & Goodhart, induce males of the same species to spawn (Burdon-Jones, 1951), while the spawning of a male sea-urchin may stimulate every other male urchin in an aquarium tank to start shedding its spermatozoa, to the dismay of the biologist hoping to work with this material. This phenomenon is put to commercial use in the oyster industry, the gonads of some hundreds of oysters being thrown into oyster-beds to stimulate mass spawning, and thereby increase the oyster population (Quayle, 1940). So far as the induction of spawning is concerned, the nature of the responsible substances has been mainly investigated in oysters, in particular by Nelson & Allison (1937), Galtsoff (1940) and Nelson (1941). Although the results of these studies are not particularly encouraging from the point of view of isolating one compound with specific stimulatory activity, there

TABLE 1

Sperm-egg interacting substances

Name	*Alternative name*	*Biological properties*	*Chemical characteristics*
Fertilizin	Gynogamone I (G.I)	Activates and/or stimulates homologous spermatozoa. Attracts plant, but not animal, spermatozoa.	Claimed to be echinochrome or to have echinochrome as prosthetic group in eggs of *A. lixula*, and astaxanthine in trout eggs. Claims unjustified. See chapter 4 for chemotaxis of plant spermatozoa.
Fertilizin or Isoagglutinin	Gynogamone II (G.II)	May reversibly or irreversibly agglutinate homologous spermatozoa.	Mucopolysaccharide, molecular weight, *ca.* 300,000.
Hetero-agglutinin	—	May irreversibly agglutinate heterologous spermatozoa.	Mucopolysaccharide. May not be chemically distinct from fertilizin.
Egg-antifertilizin	—	Neutralises fertilizin. Forms precipitation membrane at surface of egg jelly.	Protein.
	Androgamone I (A.I)	Inhibits activity of spermatozoa. Present in salmon, but not normally in sea-urchin semen.	Soluble in methanol; diffuses through cellophane; not inactivated by trypsin; heat resistant.
Sperm-antifertilizin	Androgamone II (A.II)	Neutralises fertilizin. Forms precipitation membrane at surface of egg jelly (see Egg-antifertilizin).	Protein. Properties disputed.
Sperm Lysin (1)	Androgamone III (A.III)	Effect on surface of sea-urchin egg similar to bee venom and detergents. Haemolytic action.	Soluble in methanol; diffuses through cellophane; not inactivated by trypsin; probably a long chain unsaturated fatty acid.
Sperm Lysin (2) or Egg membrane Lysin	—	Dissolves membranes round unfertilized eggs.	Protein. Similar to Hyaluronidase. (A.II also claimed to act like Hyaluronidase).
Hyaluronidase	—	Depolymerises hyaluronic acid.	Protein.

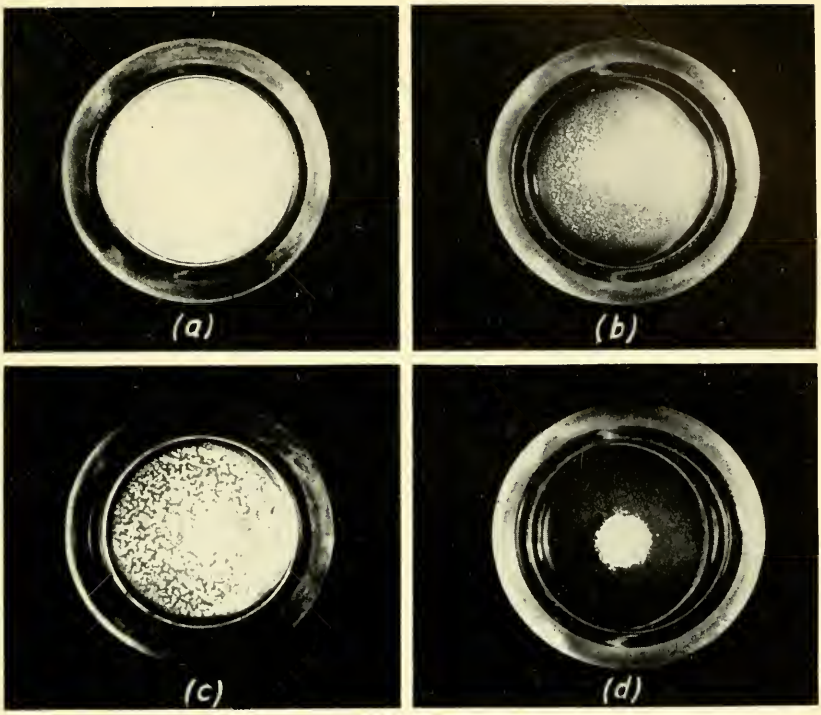


PLATE III

Agglutination of a 2% suspension of spermatozoa of *Megathura crenulata* in the presence of homologous egg water. *a*, untreated; *b*, 15 secs. after addition of egg water; *c*, after 30 secs.; *d*, after 10 min. Magnification, $\times\frac{1}{2}$. Tyler (1940*b*).

is evidently something in the ejaculates of many male and female invertebrates which stimulates other members of the same species to shed their gametes; and it is in part due to the existence of this something that such intensive investigations have been made into the nature and properties of sperm-egg interacting substances.

Agglutination of spermatozoa by egg water. In the presence of water in which unfertilized eggs of the same species have been standing, 'egg water', spermatozoa come together in clusters (Plate III), which consist of dense aggregates of spermatozoa, usually stuck together by their heads, but sometimes by their tails and even, on occasions, head to tail. The phenomenon has been observed in echinoderms and annelids (F. R. Lillie, 1919), molluscs (Tyler, 1940*b*; von Medem, 1942), ascidians (Minganti, 1951), cyclostomes (Schartau & Montalenti, 1941), fish (Hartmann, 1944; von Medem *et al.*, 1949) and amphibia (Glaser, 1921) and, in some forms, is quite similar to the agglutination of bull spermatozoa in the presence of antisera (Henle *et al.*, 1938). The agglutination of sea-urchin spermatozoa is often spontaneously reversible, the time for reversal depending on the concentration of spermatozoa and of the compound in the egg water responsible for the effect. This compound is called fertilizin or Gynogamone II (G.II). It used to be thought that the unfertilized egg secreted fertilizin into the surrounding sea water. But in 1939 and 1940 Tyler & Fox showed that fertilizin was derived from the jelly which surrounds the unfertilized eggs of a number of aquatic organisms. This jelly, which Vasseur (1951) believes is secreted by follicle cells in the ovary, swells and slowly dissolves in sea water. As the dissolution proceeds, the sperm-agglutinating power, or titre, of the egg water becomes stronger. The process of dissolution can be greatly accelerated, without injury to the eggs, by acidifying the sea water, the degree of acidification required varying in different species. The sperm-agglutinating power of fertilizin so obtained is equal to, or greater than, that from untreated suspensions in which the unfertilized eggs have been allowed to remain in sea water for a sufficient time for the jelly round them to dissolve. Eggs do not usually yield any more fertilizin once the jelly round them has been removed or has dissolved. These observations have led to the view that egg jelly *is* fertilizin and, in support of this contention, Tyler (1942*a*) reported that virtually all the organic material from an electro-

phoretically homogeneous solution of egg jelly could be absorbed by spermatozoa. In spite of this evidence, Motomura (1950, 1953*a*, *b*) has claimed that a sperm-agglutinating factor, *Cytofertilizin*, can be extracted from jelly-free unfertilized eggs of *Hemicentrotus pulcherrimus* (A. Agassiz) and from the perivitelline fluid of fertilized eggs of the same species; also, that cytofertilizin is secreted, transiently, by fertilized eggs of *Strongylocentrotus nudus* (A. Agassiz) and *Temnopleurus hardwicki* (Gray). In the last two cases, the vitelline membrane was removed before fertilization by treatment of the unfertilized eggs for 10–15 minutes, with 0·5M-NaSCN, so that there was no fertilization membrane and, therefore, no perivitelline fluid at the time when the cytofertilizin was secreted. In spite of being queried by Byers (1951), Motomura's results cannot be ignored or explained away on the basis of species differences. The only way of resolving the impasse created by this work is to repeat Motomura's experiments, using his techniques but eggs of *Arbacia punctulata*, *Arbacia lixula*, *Paracentrotus lividus*, *Echinocardium cordatum*, *Strongylocentrotus purpuratus*, or *Lytechinus pictus* (Verrill). Although the agglutination of sea-urchin spermatozoa by homologous egg water is often spontaneously reversible, this is not always the case. A difficulty therefore arises in distinguishing between irreversible agglutination by homologous fertilizin or cytofertilizin, and irreversible agglutination caused by 'unnatural' substances (e.g. cerium ions, Gray, 1920), of which cytofertilizin could conceivably be an example, though this is unlikely.

The procedure for extracting fertilizin in powder form is given in Table 2 (Tyler, 1949). Defining agglutination titre as the maximum dilution at which agglutination is observed when added to an equal volume of a 1% suspension of spermatozoa (about 2.10^8 sperm/ml.), the egg water from a 20% suspension of eggs, as in Table 2, has an agglutination titre of about 1,000. Fertilizin is a hexosamine-free glycoprotein or mucopolysaccharide containing 20% protein and 80% polysaccharide esterified, according to Vasseur (1952), with one sulphate group per monosaccharide residue; * it is highly acidic, the electrophoretic mobility, about 18×10^{-5} cm^2/sec./volt, towards the anode, changing little as the pH is lowered from 8·6 to 2·0 (Runnström *et al.*, 1942; Tyler,

* The amounts of sulphate found in the molecule are not always consistent with this arrangement.

1949). These acidic properties are due to the large amount of acid-labile sulphate, which may amount to 25% or more, in the molecule. Fertilizin's sedimentation constant is $2{\cdot}9$–$6{\cdot}3 \times 10^{-13}$. Measurements of diffusion coefficients are difficult to make on fertilizin solutions, because they gelate at relatively low concentrations; but on the basis of a sedimentation constant of $6{\cdot}3 \times 10^{-13}$ and the value $2{\cdot}1 \times 10^{-7}$ cm^2/sec., which Tyler (1949) and Tyler *et al.* (1954) considered the most accurate of their estimates of the diffusion coefficient, the molecular weight of fertilizin from *Arbacia punctulata* is about 300,000. A reciprocal density of 0·65,

TABLE 2

Preparation of powdered fertilizin from sea-urchin egg water

(1) Prepare 20% suspension, by volume, of unfertilized eggs.
(2) Bring pH to 3·5 with 0·1 N-HCl.
(3) Centrifuge and remove supernatant.
(4) Add 40 ml. N-NaOH per litre of supernatant.
(5) Suspend precipitate in 3·3% NaCl and dialyze against 3·3% NaCl.
(6) Remove insoluble particles and precipitate with 1¼ vols. 95% ethanol.
(7) Dissolve in 3·3% NaCl, re-precipitate with ethanol or saturated $(NH_4)_2SO_4$, and dry.

Yield, ca. 250 mg./L. (*S. purpuratus*)

which is of the right order for a polysaccharide, is assumed in the calculations. The corresponding axial ratio (unhydrated prolate ellipsoid) is 28: 1, and, with a reasonable value for water of hydration, the ratio becomes about 20: 1.

Fertilizin with a high sulphur content, i.e. from egg suspensions of *Strongylocentrotus droebachiensis* (O. F. Müller) (9%), and *Paracentrotus lividus* (8%), gives a good agglutination reaction with homologous spermatozoa. Egg jelly of *Brissopsis lyrifera* (Forbes), which has a small sulphur content, 2·7%, does not cause agglutination of homologous spermatozoa, from which Vasseur (1952) concludes that agglutination is dependent on fertilizin containing an adequate number of sulphate groups. But we shall see, when considering the serological aspects of agglutination, that non-agglutinating fertilizin can be rendered agglutinating by the addition of adjuvants, while agglutinating fertilizin can be rendered non-agglutinating by various treatments, including trypsin (Tyler & Fox, 1940) and periodate (Immers & Vasseur, 1949), which could be interpreted as meaning that both the protein and the polysaccharide part of the molecule are necessary for agglutination, though this is not

proved. It follows that the presence or absence of the necessary number of sulphate groups may not be the only factor which determines the agglutinating powers of fertilizin towards homologous spermatozoa.

Interesting studies have recently been made, notably by Vasseur, on the specificity of the carbohydrate components in fertilizin obtained from different sea-urchins. The results are summarised in Table 3, from which it is unfortunately clear that one should not

TABLE 3

Carbohydrates in the egg jelly of certain Echinoderms

E. cordatum	Fucose	Vasseur, 1952
E. esculentus	Galactose	Vasseur, 1950
S. droebachiensis	Fucose and galactose	Vasseur, 1948; Vasseur & Immers, 1949
P. lividus	Fucose and glucose	Vasseur, 1948; Vasseur & Immers, 1949
E. parma	Fructose	Bishop, 1951
S. purpuratus	Galactose and fucose	Tyler, 1949
A. lixula	Fucose	Monroy *et al.*, 1954
H. pulcherrimus	Fucose	Nakano & Ohashi, 1954
P. depressus	Fucose	Nakano & Ohashi, 1954
A. crassispina	Fucose	Nakano & Ohashi, 1954

try to make any generalisations about the genus or species specificity of the carbohydrate components of sea-urchin egg jelly.

Hetero-agglutination. The agglutination of spermatozoa by homologous egg water has several features in common with serological reactions. For example, the reaction is specific. It is true that spermatozoa are also agglutinated by heterologous fertilizin, but this occurs mainly with closely related organisms. The reaction occasionally takes place between distantly related organisms, as in the case of the agglutination of *Nereis* spermatozoa by *Arbacia* egg water. F. R. Lillie believed that a distinction could be made between hetero- and iso-agglutination on the grounds that the former does not exhibit the phenomenon of spontaneous reversibility. This view is now known to be incorrect because iso-agglutination may be irreversible, as in the case of the spermatozoa of *Megathura crenulata* described by Tyler (1940*b*). Hetero-agglutination can, however, be distinguished from iso-agglutination in other ways. Tyler (1946, 1948) has studied the hetero-agglutination of vertebrate and invertebrate spermatozoa by blood, body fluids and sperm extracts. When fertilizin of one organism,

such as *Arbacia punctulata*, agglutinates the spermatozoa of a distantly related organism, such as *Nereis succinea*, the same reaction is elicited by blood, body fluids and sperm extracts; but when the organisms under examination are closely related, these latter substances do not agglutinate spermatozoa. A serum or body fluid may contain a large number of independently absorbable agglutinins, each of which exhibits group specificity, in the sense of reacting with particular spermatozoa. In the serum of *Panulirus interruptus* (Randall), for example, there are ten serologically distinguishable agglutinins, eight of which react specifically with the blood or spermatozoa of scyphozoans, phanerocephalid polychaets, holothurians, asteroids, ascidians, amphibians, reptiles and birds, but not with the blood or spermatozoa of, for example, anthozoans, cryptocephalid polychaets or turbellarians (Tyler & Metz, 1945). These hetero-agglutinins are all present in a protein fraction which is homogeneous on the basis of electrophoresis and ultracentrifuge measurements. In the same way, parts of the fertilizin molecule may carry such hetero-agglutinating properties. F. R. Lillie also based his view that the hetero-agglutinating power of egg water was due to the presence in it of a substance distinct from iso-agglutinin on the observation that the hetero-agglutinin in the egg water of *Arbacia punctulata* could be removed by spermatozoa of *Nereis succinea*, though after the removal, the egg water would still iso-agglutinate *Arbacia* spermatozoa. According to Tyler (1948), the correct interpretation of this experiment is that the fertilizin molecule has *both* hetero- and iso-agglutinating properties and that the inactivation of some molecules in a solution through a hetero-agglutination reaction would not make much difference to the iso-agglutination titre of the solution. A similar situation exists in human blood sera which contain hetero-agglutinins for the red blood cells of other vertebrates, though they may not be distinct from the iso-agglutinins which distinguish the blood groups.

To sum up this section, the reactions of fertilizin are dominantly species specific; cross reactions occur mainly with spermatozoa of closely related organisms. The occasional cross reactions with spermatozoa of remotely related organisms are due to the similarities between certain molecular configurations in the fertilizin molecule and those in other proteins in the body fluids or blood of the organism in question.

Multivalent and univalent fertilizin. Tyler (1948) has put forward the view that agglutination is caused by the combination of fertilizin molecules which, like antigens and antibodies, are 'multivalent' * with respect to their combining groups, with spermatozoa which are multivalent with respect to the number of receptor groups on their surfaces. If, therefore, a fertilizin molecule is to cause sperm agglutination, it must have at least two combining groups so that two spermatozoa can become attached to it. If one spermatozoon with two or more receptors on its surface combines with two or more fertilizin molecules and each multivalent fertilizin molecule combines with a number of spermatozoa, the observed macroscopic agglutination will occur. Once spermatozoa have reversibly agglutinated, they cannot be re-agglutinated and their fertilizing capacity is reduced. Tyler (1941*a*) has suggested that the reversal of agglutination may be due to the fertilizin molecules being split into 'univalent' fragments by an enzyme in the spermatozoa, or possibly by the movements of the spermatozoa themselves. If the former hypothesis is correct, each spermatozoon will be covered by univalent fragments and therefore cannot be re-agglutinated by fresh fertilizin. In spite of certain obvious differences, the agglutination of vertebrate blood cells by influenza and other viruses is a rather similar phenomenon as the reaction is spontaneously reversible, after which the blood cells cannot be re-agglutinated (Hirst, 1942). This haemagglutination reaction is inhibited by a number of cell-free extracts and tissue fluids and there is some evidence that the reactive groups of the inhibitors and the blood cells are mucopolysaccharides. The receptor groups are sensitive to periodate, as is fertilizin (Vasseur, 1952). In support of the multivalent fertilizin theory, Tyler (1941*a*, 1942*a*) showed that treatment of fertilizin with proteolytic enzymes, ultraviolet light and heat made it incapable of causing sperm agglutination, though spermatozoa treated with this modified fertilizin could not be agglutinated by normal fertilizin and sustained a loss of fertilizing capacity without impairment of motility. (Untreated fertilizin also reduces the fertilizing capacity of homologous spermatozoa.) Tyler suggested that the above treatments of fertilizin converted it into a univalent form. The spermatozoa com-

* The Lattice theory of antigen–antibody combination requires the existence of divalent or multivalent antibodies; but the existence of such antibodies has not yet been unequivocally demonstrated.

bine with univalent fertilizin and therefore sustain a loss in fertilizing capacity because a certain number of combining groups on their surfaces, which would otherwise be used during the union of the spermatozoa with homologous eggs, are covered by fertilizin molecules. No agglutination occurs because the fertilizin molecules, being univalent, only have one combining group per molecule. The univalent fertilizin concept provides an explanation of the apparent absence of fertilizin in many organisms. The egg water of *Cumingia tellinoides* (Sampson, 1922), of *Urechis caupo* (Tyler, 1941*a*) and of the starfish *Patiria miniata* (Brandt) (Metz, 1945) does not cause agglutination of homologous spermatozoa. Metz was able to make these starfish spermatozoa agglutinate in the presence of egg water by the addition of an adjuvant, hen's egg-white, which, in the absence of starfish egg water, did not cause sperm agglutination. Again, there is an analogy in the field of Rh antibodies. These are quite often found in a non-agglutinating, univalent form and can be made to agglutinate Rh-positive cells by such substances as serum albumin (Race, 1944; de Burgh, *et al.*, 1946). Furthermore, multivalent immune antibodies can be converted into univalent forms by treatments similar to those which have been used on fertilizin (Tyler, 1945).

Zone phenomenon. The resemblance between the behaviour of spermatozoa in the presence of fertilizin and serological reactions is strengthened by the occurrence of the zone phenomenon (Tyler, 1940*a*). In serological reactions the zone phenomenon refers to the fact that maximum or most rapid agglutination or precipitation only occurs when antigens and antibodies are mixed in particular proportions. The zone phenomenon provides an interpretation of the conditions in which feeble sperm agglutination is observed, for if fertilizin molecules are present in great excess, there will be enough of them to saturate all the combining groups on individual spermatozoa without the necessity for sharing of fertilizin molecules by numbers of spermatozoa, with consequent agglutination. Alternatively, the intensity of the agglutination reaction may be reduced if univalent as well as multivalent fertilizin molecules are present in egg water and the former are nearly able to saturate the combining groups on the spermatozoa. Zone phenomena have been observed and studied in the spermatozoa of *Lytechinus pictus* by Spikes (1949*a*).

Role of egg jelly in fertilization. Although the description of the

reaction between spermatozoa and egg water as a typical serological reaction is plausible—fertilizin can even combine with complement (Tyler, 1942*b*)—criticisms of the analogy have not been lacking, while some of the difficulties will not have escaped the reader. Rybak (1949), for example, said that reversal of agglutination was not due to the splitting of fertilizin but to a change in the spermatozoa. Popa made a similar claim as long ago as 1927 and more recently, J. C. Dan (1952, 1955) has published electron micrographs showing morphological changes which are believed to take place after treatment of sea-urchin spermatozoa with egg water. Rybak also disbelieves in univalent fertilizin, but his experiments in support of this view are not convincing. A further difficulty is the fact that fertilizin appears to *impair* the fertilizing capacity of spermatozoa by masking some of the combining groups on the sperm head surface. It seems curious that an unfertilized egg should be normally surrounded by a substance which positively interferes with fertilization. At the same time Tyler (1941*a*) showed that following complete removal of jelly from unfertilized eggs of *Strongylocentrotus purpuratus*, by methods which did not harm the eggs from the point of view of subsequent development, more spermatozoa were needed to achieve a particular percentage of fertilized eggs than when the jelly was present. This observation was confirmed and put on a quantitative basis, in terms of the probability of successful sperm-egg collisions, by Rothschild & Swann (1951).

An interesting question has been raised by Monroy *et al.* (1954) —whether the reaction between a spermatozoon and egg jelly in solution in sea water, or between a spermatozoon and egg jelly surrounding an egg, are necessarily the same, a subject which requires further investigation. As regards the former reaction, these workers, together with Hultin *et al.* (1952), have shown that living spermotozoa of *Arbacia lixula* and *Echinocardium cordatum* remove fucose from egg water (Table 4), which confirms that surface groups on the spermatozoa react with and bind fertilizin. (Mudd *et al.* (1929) found that the ζ-potential of the spermatozoa of *Arbacia punctulata* increased from -22 to -25 mV. in the presence of homologous egg water.) Monroy, in the paper referred to above, and Vasseur (1952) suggest that, as alkylation of amino groups on spermatozoa inhibits agglutination (Metz & Donovan, 1951), the reaction given in Table 4 is between amino groups on sperm

surfaces and sulphate groups in fertilizin. Attractive as this idea may be, it is probably an over-simplification, if only because steric factors play a dominating role in the specificity of antigen-antibody reactions and, by analogy, might be expected to be important in sperm agglutination.

There are, of course, aquatic organisms whose unfertilized eggs are not surrounded by an obvious shell of jelly. The best known example is that of *Nereis succinea*, though there is no doubt that a substance with similar properties to sea-urchin egg jelly diffuses

TABLE 4

Effect of homologous spermatozoa on fucose content, in μg/ml., of egg water (A. lixula), *Monroy* et al. (*1954*)

Without sperm	*After treatment with sperm*
234·0	44·0
9·8	4·2
120·0	49·0
19·8	2·2
42·0	25·6

out of unfertilized *Nereis* eggs. These eggs extrude jelly, which has fertilizin-like properties, after fertilization.

One obvious function of egg jelly in fertilization is to prolong the life of the spermatozoon. This effect is not very specific and can be reproduced by adding albumin to the sea water in which the spermatozoa are suspended (Wicklund, 1954). As chelating agents such as versene have the same effect (Tyler, 1953; Rothschild & Tyler, 1954), it may be that, in this context, egg water protects the spermatozoa from the adverse effects of small quantities of heavy metals in sea water. Two lines of attack on the problem of the reactions between spermatozoa and fertilizin might repay further investigation: first, a careful investigation with the electron micrograph into the morphology of spermatozoa before and after treatment with multivalent and univalent fertilizin. Care must be taken in such experiments to resist the temptation to select and generalise from photographs which show interesting structural changes, when others from the same suspension do not. The spermatozoa of *Nereis succinea* would be particularly suitable for such a study as Lillie (1919) noted that the heads of agglutinated *Nereis* spermatozoa are swollen and spherical. Secondly,

the fertilizing capacity of spermatozoa after subjection to *different* concentrations of multivalent and univalent fertilizin could be quantitatively examined with advantage.

Activation of spermatozoa by egg water. Egg water makes homologous spermatozoa move more actively, respire at a greater rate, and continue their movement, and therefore their respiration,

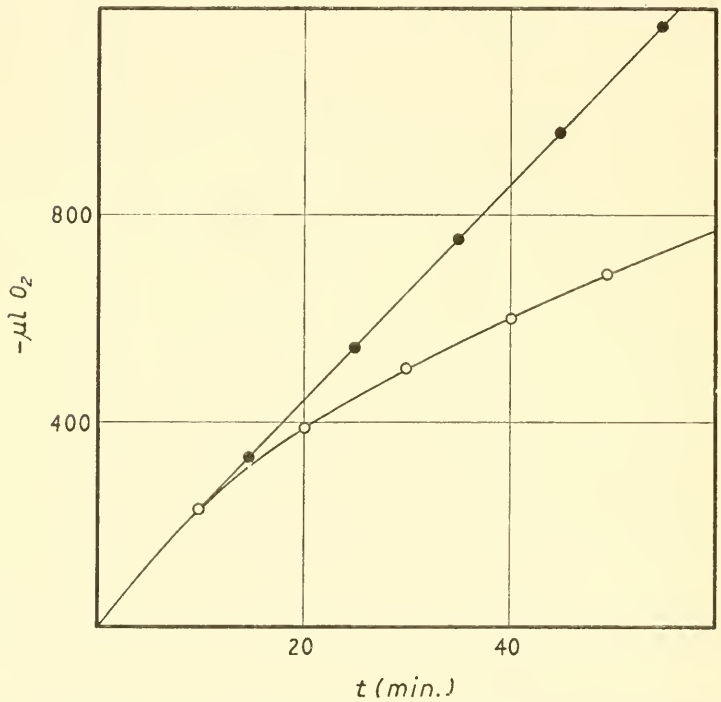


FIG. 7.—Effect of egg secretions on the O_2 uptake of sea-urchin spermatozoa (*Psammechinus miliaris*). ●, with egg secretions; ○, without egg secretions. After Gray (1928).

for longer than they do in ordinary sea water. The effect is rather variable, though when it is present, there is no doubt about its existence. Gray (1928) was the first to examine this phenomenon quantitatively, using the spermatozoa of *Psammechinus miliaris* (Fig. 7). He found that in some cases, the initial rate of O_2 uptake was four times as great in egg water as in sea water. The prolongation of sperm movement has obvious advantages from the point of view of fertilization, though without a detailed knowledge

of the morphology of the movement, it is not so obvious that an increase in speed is beneficial in increasing the chance of an encounter between a spermatozoon and an egg. This question, which involves consideration of the statistical mechanics of sperm movement, is gone into in greater detail in chapters 4 and 9. An increase in sperm respiration in the presence of egg water does not always occur. Hayashi (1946), Spikes (1949*b*) and Rothschild (1952) observed a decrease in O_2 uptake on addition of egg water, which was not due to agglutination, while Carter (1931) found that fully ripe spermatozoa of *Echinus esculentus* and *Psammechinus miliaris* appeared to be unaffected by egg secretions. On the other hand, Vasseur (1952) has shown that egg water undoubtedly increases the O_2 uptake of ageing sperm suspensions of a number of different genera and species of sea-urchins. In the Mediterranean sea-urchin, *Arbacia lixula*, the substance, sometimes called Gynogamone I (G.I), responsible for sperm activation, was said by Hartmann *et al.* (1940) to be the substituted naphthoquinone, echinochrome (2-ethyl-3, 5, 6, 7, 8-pentoxinaphthoquinone-1, 4), probably in equilibrium with isomeric quinones. According to Hartmann and his co-workers, echinochrome, which is responsible for the pink colour of these sea-urchin eggs, is bound to a protein carrier within the eggs. In this form echinochrome is biologically inactive and before being able to activate spermatozoa, it must become attached to a second carrier derived from the egg jelly, or be separated from its original protein carrier. The ternary complex is said to 'activate' spermatozoa at a dilution of $1/3.10^{11}$. The significance of the word 'activate' is not clear in this context and if it merely means that the spermatozoa seemed to move more quickly in the presence of the ternary complex, the observation should be taken with a grain of salt. No quantitative information, based on O_2 uptake, is available. A similar claim, that astaxanthine, which is normally present in trout eggs, increases the activity of trout spermatozoa, was made by Hartmann *et al.* (1947). As this is not a book about spermatozoa *per se*, the differences of opinion which exist about the effects of echinochrome and many other substances on the activity and metabolism of spermatozoa will not be further discusssed. Further information will be found in Mann's recent book, *The Biochemistry of Semen* (1954), an important paper by Bielig & Dohrn (1950) which casts a good deal of doubt on the echinochrome story, and reviews by Tyler (1948) and

Rothschild (1951). To sum up, there is no doubt that egg water does in certain circumstances stimulate spermatozoa to increased and more prolonged movement and metabolism. Whether this is due to a substance which is not the same as fertilizin is an open question. The protein carrier and ternary complex story should not be accepted until it is independently confirmed.

Inhibition of sperm agglutination by egg and sperm extracts. F. R. Lillie suggested in 1914 that a substance which could neutralise fertilizin was present in sea-urchin eggs. This substance, called antifertilizin or egg-antifertilizin, was extracted from sea-urchin eggs by Runnström (1935*b*) and Tyler (1940*a*). Using the gamone terminology, antifertilizin would be called G.III or, if one wanted to be pedantic, Gynandrogamone I, as it can be extracted from eggs and spermatozoa. Both egg- and sperm-antifertilizin inhibit the action of egg water and, in suitable conditions, precipitate fertilizin; they make unfertilized eggs of the same species agglutinate and induce the formation of a membrane-like structure, usually known as a precipitation membrane, on the surface of egg jelly. The latter is somewhat similar to the Neufeld reaction of pneumococci and other encapsulated bacteria reacting with specific antisera. If fresh eggs are extracted by freezing and thawing, no antifertilizin is obtained, because it is neutralised by the fertilizin in the jelly present round such eggs. Injection of egg-antifertilizin into rabbits induces the production of antibodies which precipitate antifertilizin and agglutinate homologous spermatozoa (Tyler, 1948, p. 202).

A substance with very similar properties can be extracted by heating (Frank, 1939), freezing and thawing (Tyler, 1939), and acidification (Tyler & O'Melveny, 1941) from spermatozoa, Table 5; it also is called antifertilizin and is the substance with which fertilizin is believed to combine, causing agglutination. It is evident from what has been said immediately above that there is an antigenic complementarity between sperm-antifertilizin and the antibodies formed in rabbits following injection of egg-antifertilizin.

There has been some discussion as to whether sperm-antifertilizin is an acidic protein, as stated above, or, as Hultin (1947) thinks, an unspecific basic protein derived from sperm nuclei. The latter induces the formation of a precipitation membrane on egg jelly and causes homologous sperm agglutination (Metz, 1949),

while sperm-antifertilizin does not cause homologous sperm agglutination. This complicated question, which has been discussed at great length by Runnström (1949), is not yet resolved, and further work is needed to clarify the chemical properties of sperm-antifertilizin, which is certainly of biological importance, both in eggs and spermatozoa. If a decision had to be taken now, it would probably be in favour of sperm-antifertilizin being an acidic protein, and not an unspecific basic protein.

F. R. Lillie believed that the function of egg-antifertilizin was to neutralise fertilizin which would otherwise diffuse out of the egg

TABLE 5

Electrophoresis of purified solutions of sperm-antifertilizin of E. cordatum (*Runnström* et al., *1942*) *and* L. pictus (*Tyler, 1949*)

pH	*Buffer*	*Mobility in cm.²/sec./volt* . 10^5	
		E. cordatum	L. pictus
4·0	Acetate	3·0	
4·5	Acetate		4·2
4·6	Acetate	3·7	
4·9	Acetate		4·8
5·0	Acetate	4·1	
6·0	Phosphate		6·1
6·9	Phosphate	6·4	
7·3	Barbital		9·3

at a time when there would be no point in it doing so. This idea loses much of its value now that we believe fertilizin is in general derived from egg jelly and not from eggs; and it is therefore not surprising that Monroy & Runnström (1950) were able to extract antifertilizin from fertilized eggs. Runnström (1952) believes that egg-antifertilizin is a chromoprotein which reacts with the vitelline membrane on the unfertilized egg at the moment of fertilization, hardening and converting it into the fertilization membrane. If this view is correct, egg-antifertilizin might be located in the cortical granules of the egg, as these are known to have a hardening or tanning effect on the fertilization membrane (see pp. 9–10).

Sperm lysins. Spermatozoa contain compounds which can break down or dissolve the membranes and jelly which so often surround unfertilized eggs, and a similar substance may have a lytic effect on the plasma membrane itself. Some such action seems to be required at fertilization, as the spermatozoon has got to get through

the plasma membrane after attachment to the egg surface. Two different compounds with these properties can be extracted from spermatozoa, though there is no evidence that both can be obtained from sperm of the same species. The existence of these two classes of compounds, both of which are called sperm lysins in the literature, makes the terminology confusing. One of them, sometimes called Androgamone III (A.III), but more often sperm lysin, was extracted with methanol from lyophilized sea-urchin spermatozoa by Runnström *et al.* (1945). It diffuses through cellophane and is therefore probably not a protein. It may be a fatty acid. A similar substance, which is believed to be an 18-carbon fatty acid with four double bonds, can be extracted from mackerel testes. This lysin, which is found in the supernatant fluid after spermatozoa have been centrifuged, is haemolytic and activates unfertilized eggs, though it inhibits fertilization. Its effects can be reproduced by detergents and bee venom. Osterhout (1950, 1952) has obtained a similar substance by heating the spermatozoa of *Nereis succinea* for ten minutes at 55° C. The compound released into the sea water by this treatment is highly surface active and activates eggs of the same species. Similar results are obtained with the detergent Duponol, which is mainly composed of sodium dodecyl sulphate. Some caution is necessary in the interpretation of such experiments, as Osterhout (1953) has also shown that the same treatment displaces protamine from *Nereis* spermatozoa, though it appears from his brief note that the detergent-like compound is responsible for the activation of eggs of the same species, rather than protamine.

The other compounds with lytic properties, which can be extracted from spermatozoa, are proteins. The most famous of these is the enzyme hyaluronidase which causes dispersal of the follicle cells that surround unfertilized mammalian eggs. Something similar to hyaluronidase is believed to exist in the spermatozoa of *Discoglossus pictus* Otth (Hibbard, 1928; Parat, 1933), and of marine invertebrates. Hartmann & Schartau (1939) were the first to make this claim as regards sea-urchin spermatozoa and Monroy & Ruffo (1947) said that they also could extract a substance from these spermatozoa, by treatment with 0·1N-acetic acid followed by precipitation with acetone, which 'completely dissolved' (p. 604) the jelly round unfertilized eggs. There is some doubt about these claims because sperm-antifertilizin sometimes has a curious

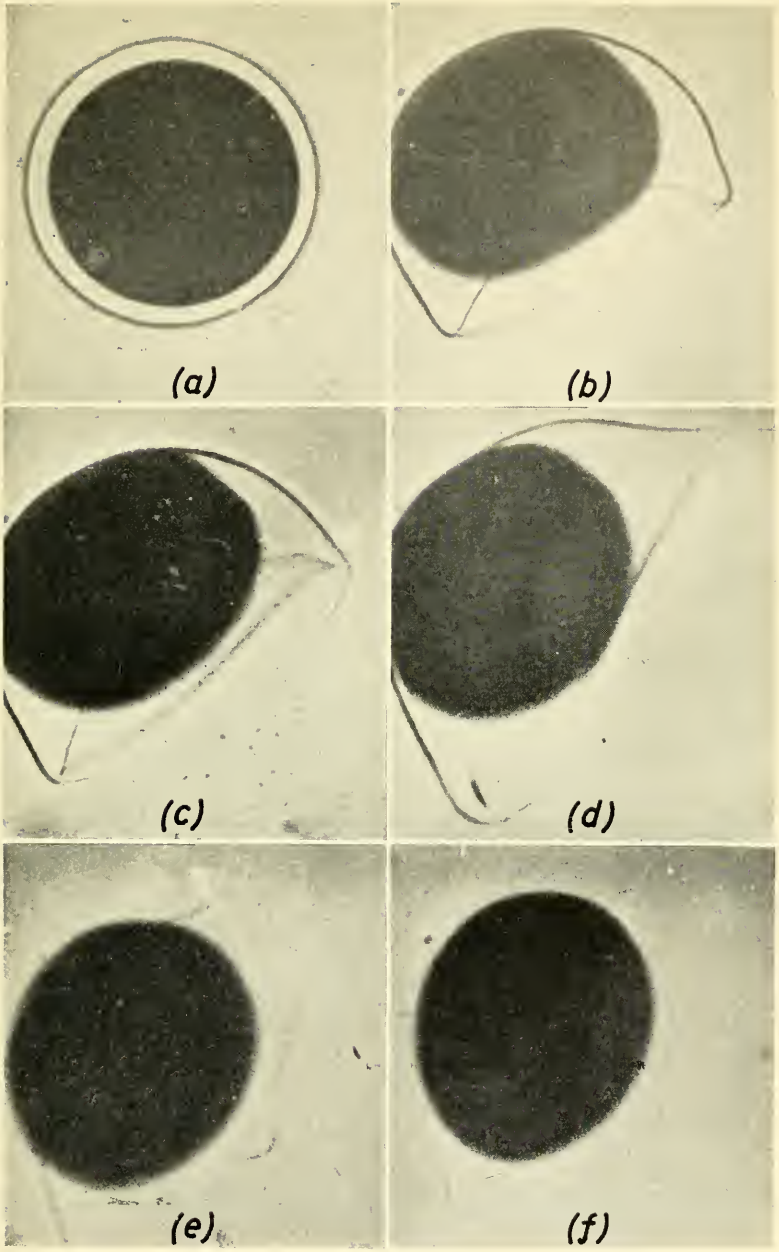


PLATE IV

Dissolution of egg membrane of *Megathura crenulata* by an extract of a 1% homologous sperm suspension. *a*, 1 min., *b*, 1·75 min., *c*, 2·00 min., *d*, 2·25 min., *e*, 3·00 min., *f*, 3·25 min. after addition of extract. Photograph by A. Tyler.

effect on egg jelly, apart from forming a precipitation membrane at its surface. After the precipitation membrane has formed, the jelly contracts to such an extent that its surface becomes contiguous with that of the egg proper and is, consequently, invisible. On the basis of this phenomenon Tyler & O'Melveny (1941) believe that the substance claimed to have hyaluronidase activity is in reality sperm-antifertilizin and that sea-urchin spermatozoa do not contain a separate depolymerase. The experiments of Krauss (1950) confirm that claims for the existence of a hyaluronidase-like enzyme in sea-urchin spermatozoa should not be accepted. Vasseur (1951) also reported that sea-urchin spermatozoa contained an enzyme which splits egg jelly. But in this case again, the claim has been refuted, by Monroy & Tosi (1952), who also cast doubt on the earlier work on this subject, mentioned above, by Monroy & Ruffo (1947). Ishida (1954) has recently claimed that sea-urchin spermatozoa release a jelly-dissolving substance at the moment of fertilization. The situation is sufficiently confused to merit systematic re-examination.

A protein which is, however, distinct from sperm-antifertilizin can be extracted from limpet spermatozoa. The unfertilized egg of *Megathura crenulata* is surrounded by a tough membrane which is not dissolved by concentrated acid applied over a period of hours. Yet suspensions of homologous spermatozoa dissolve the membrane in a matter of minutes, Plate IV, while extracts obtained by freezing sperm suspensions to —80° C and thawing achieve the same result. These eggs also have jelly round them, through which the spermatozoa must get to reach the membrane (Tyler, 1939). Berg (1950*a*) has made similar observations on the eggs of *Mytilus edulis* and Monroy (1948) on those of *Pomatoceros triqueter*. One cannot help feeling that, although enzymes of this type may dissolve membranes external to the egg surface, they must also have a function in softening up the plasma membrane, so that the spermatozoon is able to enter the egg and get on with the business of syngamy.

Inhibitors of sperm movement derived from spermatozoa or semen. A substance which inhibits sperm movement and might, therefore, be said to antagonize fertilizin, can be extracted from sea-urchin spermatozoa. This substance, Androgamone I (A.I), can be extracted from these spermatozoa by a variety of treatments such as centrifugation (Southwick, 1939; Vasseur & Hagström, 1946; Rothschild, 1948), warm water (Frank, 1939), or extraction with

methanol (Runnström *et al.*, 1944*a*). Whether this substance normally diffuses out of sea-urchin spermatozoa and therefore has a function in fertilization is much more debatable. According to Hartmann *et al.* (1940), A.I. is responsible for the lack of sperm movement in undiluted sea-urchin semen. This is wrong as the lack of movement has been proved to be due to lack of oxygen; sea-urchin spermatozoa can be induced to move in undiluted semen by increasing the oxygen tension, while the effect can be reversed by replacing the oxygen with nitrogen (Rothschild, 1948). Spermatozoa in salmon semen cannot be induced to move by increasing the oxygen tension (Rothschild, 1951); there is no doubt that salmon semen contains an inhibitory substance and that it has a genuine biological function (Runnström *et al.*, 1944*a*). When ejaculated salmon semen is diluted with water, the concentration of the inhibitory substance, which is not a protein and diffuses through cellophane, falls below the level at which it inhibits movement, and the spermatozoa become motile.

Although the systematic study of sperm-egg interacting substances was started by F. R. Lillie (1919) * and Just (1939),* intensive investigations into their biological and chemical properties only started in about 1939. Like all young subjects, this one is still confused and therefore difficult to expound in an aesthetically satisfying way. Some of the more 'straightforward' aspects are discussed in the succeeding chapters. The reader may be surprised that, apart from studies on hyaluronidase, so much of this work has been done on marine invertebrates. Is there, for example, any similarity between the causes of sperm inactivity in the mammalian epididymis and the causes of sperm inactivity in undiluted salmon semen? Apart from Glaser's preliminary observations (1921) on frog spermatozoa, are there biological similarities between the jelly round frogs' eggs, whose chemistry has been intensively studied (Folkes *et al.*, 1950), and fertilizin? Does it, for example, inhibit the clotting of blood? Such questions might repay investigation.

* These contain references to early work on this subject.

CHAPTER 3

SPERM-EGG INTERACTING SUBSTANCES, II

Morphology of chemotaxis. This chapter is concerned with the chemotaxis of spermatozoa towards eggs, as a result of the secretion of substances from eggs or the cells near them. Except possibly in the case of the coelenterate *Spirocodon saltatrix* (J. C. Dan, 1950*b*), the phenomenon almost certainly does not occur, and certainly has not been shown unequivocally to occur, in the animal kingdom. But in plants and, in particular, in the ferns, mosses, horse-tails, liverworts and quillworts, the chemotaxis of spermatozoa towards egg secretions is an established fact. Chemotaxis means that spermatozoa are attracted towards eggs through the medium of some substance produced by the eggs or cells near them. The most famous case of sperm chemotaxis, discovered by Pfeffer (1884), occurs in the ferns. For example, the archegonia of bracken, *Pteridium aquilinum* (Linn.), are said to produce L-malic acid * which diffuses into the external aqueous medium. Bracken spermatozoa are sensitive to the malic acid *gradient* produced by the diffusion of this acid out of the eggs, and swim towards the source. If there were no gradient, that is if the spermatozoa were suspended in a uniform solution of malic acid, the spermatozoa could not swim preferentially in any particular direction and would remain uniformly distributed. Before considering the chemistry of chemotaxis, it is worth making a brief examination of the morphology of the reaction. The behaviour of bracken spermatozoa in ordinary tap water is shown in Fig. 8*a*. This diagram was obtained by taking a cinematograph film of the spermatozoa swimming in tap water, projecting the film frame by frame on to paper, and joining up the consecutive positions taken up by each spermatozoon by straight lines, so that every spermatozoon in the field is associated with a 'track'. The movements of the spermatozoa will be seen to be random, in the sense that the tracks do not point in any

* Although it is virtually certain that the substance *is* L-malic acid, chemical identification has not yet been achieved. Prof. E. C. Slater and I tried, unsuccessfully, to inhibit the reaction with malic dehydrogenase; but there was evidence that the enzyme preparation was not sufficiently strong to decompose the malate at the required rate.

particular direction. After the photographs on which the tracks were based had been taken, a glass pipette, filled with a 1% solution of sodium L-malate in tap water (containing 1% agar), was inserted into the sperm suspension. Malate ions immediately began to diffuse out of the pipette into the external medium; capillary effects or hydrodynamic flow, which would have confused the issue, were prevented by the agar gel. The response of the spermatozoa to the malate gradient is shown in Fig. 8*b*, which

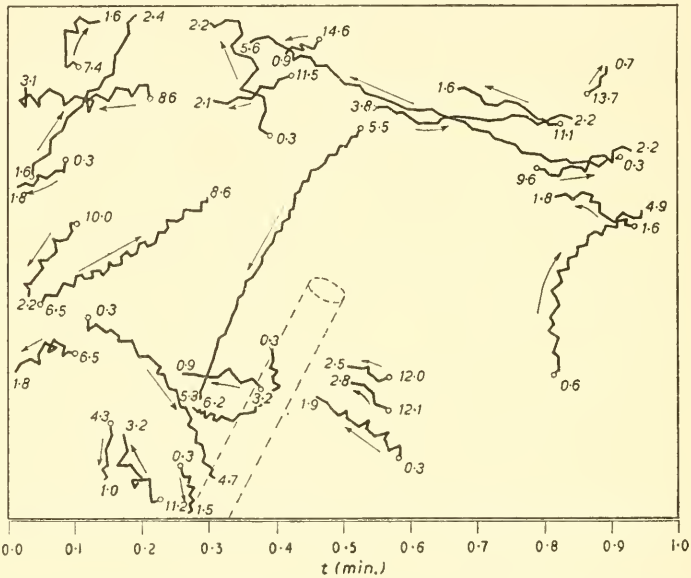


FIG. 8*a*.—Movements of bracken spermatozoa in tap water. The circles indicate where each track begins. Numbers at the beginning and end of each track refer respectively to time of start and duration of track in seconds.

requires two comments. First, the spermatozoa move in a remarkably purposeful way towards the source of malate. They do not drift statistically towards the source, as might be expected if the mechanism of attraction were of the type known as Klinokinesis with Adaptation, which occurs when Ullyott's 'Turning Worm' is subjected to a light stimulus (1936). The conclusion is almost inescapable that these spermatozoa have 'sense organs', or their functional equivalents; but in this case the sense organs must be efficient, because, in such a system, the differences in concentration between the front and back ends of the head of a sperma-

tozoon are only about 0·5% (Rothschild, 1951). If the tails of the spermatozoa are concerned with the identification of gradients, the perception system need not be so efficient, as they may be ten times as long as the head. A defect in the experiment depicted in Fig. 8*b* concerns the behaviour of the spermatozoa immediately after the pipette is inserted into the suspension. If the directions of movement of the spermatozoa are random before the pipette is

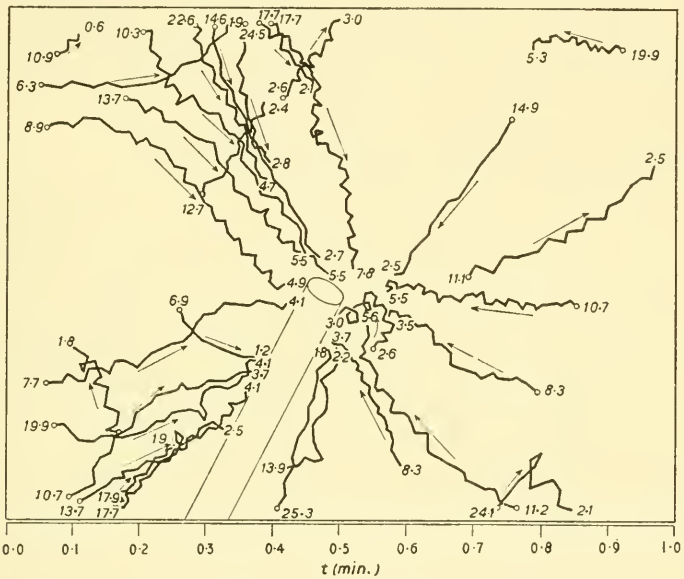


FIG. 8*b*.—Movements of bracken spermatozoa after insertion of pipette (diameter 30 microns), containing 1% sodium L-malate in a 1% agar-tap water gel, into the same sperm suspension as in Fig. 8*a*. Numbers at the beginning of tracks indicate time in seconds after insertion of pipette, Rothschild (1952).

inserted, as in Fig. 8*a*, they should be random immediately after the pipette is put in, before the malate has had time to diffuse significantly into the external medium. Unfortunately, the insertion of the pipette into the suspension inevitably causes macroscopic disturbances in the fluid, so that the earliest phases of the reaction are lost. When someone can devise a method of introducing a source of malate into a suspension without causing such disturbances, we shall learn more about the morphology of chemotaxis. One interesting question which may be resolved by such a study concerns the morphology of turning. Although there are references in the literature to spermatozoa turning and bending

towards regions of higher concentration of an attractive substance, it is not at all clear how they do it. Does the head move to the right or the left; or can the waves which travel along their tails be bilaterally asymmetrical when necessary? Or in the multiflagellate spermatozoa of ferns and mosses, do some of the flagella move more quickly than others under the appropriate conditions of stimulation? Once the spermatozoa have arrived at the source, i.e. at the tip of the pipette filled with agar and 1% sodium malate, they must be continually turning; otherwise they would not buzz round the tip like a swarm of bees, something which every student of the phenomenon has seen. The second point to notice in Fig. 8*b* is that one spermatozoon at least, at about 2·30 o'clock (11·1), was completely unaffected by the malate gradient.

Several workers have wrongly assumed that a substance which makes spermatozoa swim more quickly, according to its concentration, will act as an attractive agent. The argument is that if a spermatozoon happens to be swimming in the direction of ascending concentration of the stimulating substance, it will swim more quickly and get nearer the source of the substance. If, on the other hand, a spermatozoon happens to be swimming in the direction of descending concentration of the stimulating substance, it will swim more slowly and therefore get less far from the source. This argument is fallacious, as can be seen from the following over-simplified example. Suppose we have a suspension of spermatozoa, moving at random (Fig. 8*a*), and we suddenly point a death ray at a small region in the suspension. Any spermatozoon which happens to swim into this region will be killed, that is to say its movements will be greatly slowed up and stopped. As the movements of the spermatozoa are random, most of them will sooner or later enter the lethal area. In due course, therefore, nearly all the spermatozoa will be found in this region, where they move most slowly. Conversely, if the region in question makes the spermatozoa swim more quickly, it will on the average contain less spermatozoa than there are outside. This example is over-simplified, in that the lethal or stimulating region is assumed to end abruptly, and not to give rise to gradients in the extra-regional space. But given that this mechanism (Orthokinesis) will not achieve the desired effect and that Klinokinesis with Adaptation * is inconsistent with the

* Orthokinesis with Adaptation can also act as an attractive mechanism, though no-one has so far postulated its existence in biological systems. It suffers from

morphology of the phenomenon, we are driven to postulate the existence of sperm sense organs not fundamentally different from those which cause a moth to fly towards a light. Another example of the 'repulsive effect' of an increase in speed is a sealed test-tube containing a gas, one end of which is heated. The highest concentration of gas molecules will be found at the cold end of the test-tube, where the molecules will be moving more slowly than at the hot end.

Chemistry of chemotaxis. This subject has been most systematically examined in the ferns, horse-tails and quillworts; the

TABLE 6

Effect of certain organic acids on the spermatozoa of Equisetum arvense

L-malic	Mesotartaric	Succinic	Monobromosuccinic	Dibromosuccinic	Isodibromosuccinic	D-tartaric	Aspartic	
COOH	COOH	COOH	COOH	COOH	COOH	COOH	COOH	C^1
HO·C·H	H·C·OH	H·C·H	H·C·H	H·C·Br	H·C·Br	H·C·OH	H·C·H	C^2
H·C·H	H·C·OH	H·C·H	H·C·Br	H·C·Br	Br·C·H	HO·C·H	H·C·NH_2	C^3
COOH	COOH	COOH	COOH	COOH	COOH	COOH	COOH	C^4
+	+	o	o	o	o	o	o	

principal results are summarised in Table 9 at the end of this chapter. In this table, '+' means 'attracts', 'o' means 'does not attract', and '—' means 'no information'. The organic anion is responsible for the attraction, because in those cases where it occurs, the same effects are obtained, for example, with the sodium salt. The case of *Equisetum arvense* Linn. is in some respects the simplest, from the point of view of the specificity of the perception mechanism. The relevant information is extracted in Table 6, from which the following conclusions can be drawn: to attract the sperm of *Equisetum*, the substance must (1), be a 4-carbon dicarboxylic acid; (2), have an OH group on C^2 or C^3 (Geneva system of

the same defects as Klinokinesis with Adaptation so far as sperm chemotaxis is concerned. A few observations about the possibility of Orthokinesis with Adaptation will be found in a paper by Rothschild (1952).

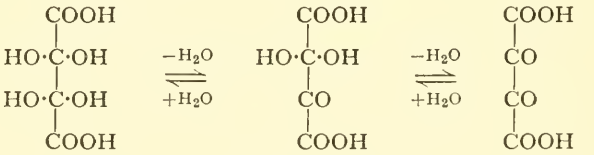
numbering the carbon atoms); and (3), have OH groups in the *cis* and not the *trans* position * on C^2 and C^3, if both C^2 and C^3 have OH groups attached to them. This information suggests other experiments to enable the perception mechanism to be examined in greater detail. Some of the more obvious substances to try are shown in Table 7. Compounds 1 and 2, Table 7, deal with the

TABLE 7

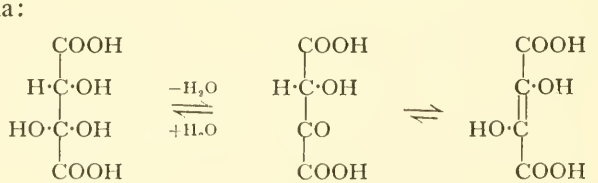
Organic acids to be tried on spermatozoa of Equisetum arvense

1.	2.	3.	4.	5.	6.
COOH HO·C·OH HO·C·OH COOH	COOH H·C·OH HO·C·OH COOH	COOH HO·C·Me H·C·Me COOH	COOH HO·C·H H·C·Me COOH	COOH HO·C·H. Me·C·H COOH	COOH Me·C·Me HO·C·Me COOH

question: does attraction occur if there are more than two OH groups on C^2 and C^3; Compound 1 is in equilibrium with diketosuccinic acid but the doubly hydrated form is believed to predominate:

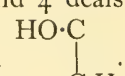
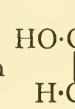


Compound 2 is equivalent to dihydroxyfumaric acid (which does not attract bracken spermatozoa), because of the following equilibria:



Compound 3 deals with the question: is the configuration HO·C·H necessary for attraction, given that the introduction of

* The use of the words *cis* and *trans* is improper, but convenient, in this context. Strictly, one should say 'have the *meso* configuration' instead of 'OH groups in the *cis* . . . position'.

a methyl group does not inhibit attraction; Compound 4 deals with the necessity or otherwise for the configuration  . The role of the configuration  is dealt with by Compound 5. If an OH group is sufficient, without an H atom on C^2 or C^3, Compound 6 should be effective. It would, perhaps, be preferable to introduce bromine atoms rather than methyl groups in Compounds 3–6 in Table 7. Unfortunately, Compounds 3 and 6 with Br atoms replacing the Me groups are too unstable to exist in aqueous solution. This type of experiment is capable of extension in many interesting directions provided the required substances can be made; but although the experiments are simple, certain precautions, which have not always been observed in the past, must be taken. As mentioned earlier, the experimental procedure is to fill a glass pipette with the test substance, put the pipette or one end of it into the sperm suspension, and see what happens. If the solution in the pipette is not a gel, spermatozoa may swim into the pipette by chance and be unable to get out, because the diameter of the pipette is too small to allow random sperm movement. Failure to appreciate this difficulty has undoubtedly been responsible for some of the claims for the existence of sperm chemotaxis in the animal kingdom. Furthermore, convection currents and hydrodynamic flow, or even a pumping and suction action, can and do occur, and may cause spurious results, both of a positive and negative kind. When using solutions of organic acids in agar gels, attention must be paid to the possibility of the acid decomposing when the agar is dissolved by heating. An alternative method of preparing the pipettes is to fill them with 1% agar in water and, after cooling, allow both ends of the pipette to dip into aqueous solutions of the organic acid. The normal process of diffusion will ultimately make the concentration of the acid in the pipette equal to that in the solutions in which the two ends of the pipette are immersed. For a pipette of length 5 cm., it will take three or four days for the average concentration in the pipette to become half of what it is in the external solutions. The process will not be seriously slowed up if carried out in a refrigerator to avoid bacterial contamination.

Returning to Table 9, a further interesting feature is the ability

of all spermatozoa except those of *Equisetum arvense* to distinguish between *cis* and *trans* unsaturated dicarboxylic acids. The spermatozoa of *Isoetes japonica* A. Braun react towards the *trans*, but are indifferent to the *cis*, configuration; while those of *Salvinia natans* Allioni, *Osmunda javanica* Blume, *Pityrogramma sulphurea* (Schwartz) and *Pteridium aquilinum*, which, with the exception of *Salvinia natans*, are ferns, are attracted by the *cis* but not the *trans* forms. Unfortunately the specificity of the reaction is not so clear in fern spermatozoa as it is in those of *Equisetum arvense*. If, for example, we examine the structure of those acids which attract the spermatozoa of *Pteridium aquilinum* (Table 8), there is evidently no single

TABLE 8

Organic acids which attract bracken spermatozoa

D-malic	L-malic	Mesotartaric	Maleic	Citraconic	Tartronic
COOH	COOH	COOH	COOH	COOH	COOH
H·C·OH	HO·C·H	H·C·OH	H·C	CH_3·C	H·C·OH
H·C·H	H·C·H	H·C·OH	H·C	H·C	COOH
COOH	COOH	COOH	COOH	COOH	

configuration which stimulates their perception mechanisms, as there seems to be in *Equisetum arvense*. In spite of these difficulties, some predictions can be made from Table 8. Other substituted and unsaturated 4-carbon *cis*-dicarboxylic acids will probably attract bracken spermatozoa. As an OH group on C^2 or C^3 appears to be essential if saturated acids are used—succinic and malonic acids do not attract—chloromalic acid would be an interesting compound to try. It is, however, difficult to predict the effect of chloromalic acid in view of the reputed behaviour of *Salvinia natans* to succinic and monobromosuccinic acids (Table 9).

In addition to their responses to organic acids, fern spermatozoa are reported to be attracted by gradients of many other substances, of which calcium, strontium, lithium, morphine and yohimbine are a few examples (Shibata, 1911). These claims must be accepted with reserve until the experiments have been repeated under care-

fully controlled conditions, particularly as a number of the reported cases of attraction are said to be associated with repulsion at the same time. It is of course possible that strong concentrations of a substance may repel and weaker concentrations of the same substance attract, organisms, as in the case of certain flagellate protozoa (Fox, 1921). But interpretation of experiments is more difficult in such cases.

This chapter is not intended to review all work on the chemotaxis of spermatozoa towards eggs, but rather to indicate future lines of research which might be profitable. There is, therefore, no discussion of sperm chemotaxis in mosses, for which sucrose, according to Pfeffer (1884), is the only attractive substance. In recent years little work has been done on the chemotaxis of plant spermatozoa, except for a few observations by the author which have been mentioned earlier, one paper by Wilkie (1954), and the work of Cook *et al.* (1948, 1951). These workers examined the chemotaxis of seaweed spermatozoa (*Fucus serratus* Linn., *Fucus vesiculosus* Linn., and *Fucus spiralis* Linn.) towards the secretions of eggs of the same species. This work is interesting from two points of view; first, the egg secretions and other substances which attract thallophyte spermatozoa are chemically quite different from those which attract fern and moss spermatozoa; secondly, in spite of an intensive investigation by organic chemists, which included the examination of sea water containing egg secretions with the mass spectrometer, it was not possible to identify the substance or substances which are produced by seaweed eggs and which attract spermatozoa of the same genus. The mass spectrometer evidence suggested that the naturally occurring substance had a molecular weight of 74; that it contained an Me group which was rather easily lost under the influence of the ion gun; that it had a carbon chain which contained three or less carbon atoms; and that it did not contain an easily lost hydrogen atom, as in an alcohol group. These considerations raised the possibility that the compound might be diethyl ether; but this compound, though it attracted seaweed spermatozoa, did not do so at the required dilution. Other substances which attract these spermatozoa are *n*-hexane, 1-hexene, *sec.*-butyl alcohol, and *n*-propyl acetate. At one moment the normally occurring compound seemed most likely to be *n*-hexane, but the data obtained with the mass spectrometer made this possibility remote.

TABLE 9

The reactions of certain plant spermatozoa towards organic acids; '+' = attracts; 'o' = does not attract; '—' = no information

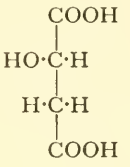
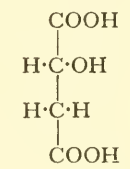
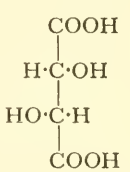
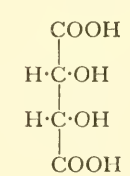
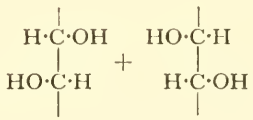
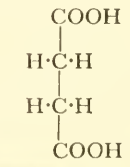
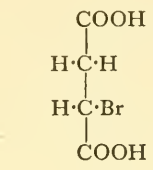
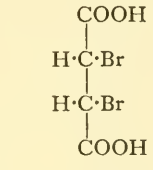
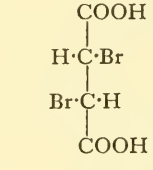
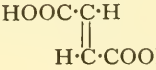
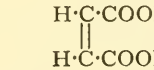
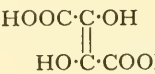
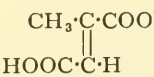
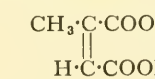
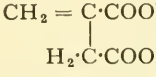
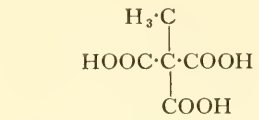
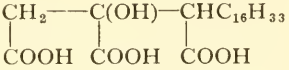
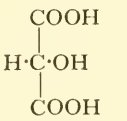
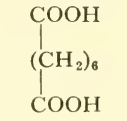
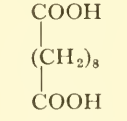
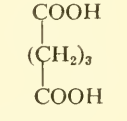
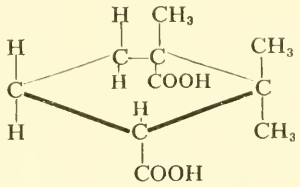
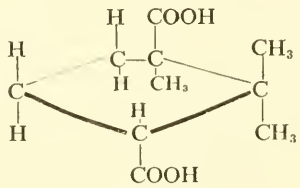
Acid	*E. arvense*	*I. japonica*	*S. natans*	*O. javanica*	*P. sulphurea*	*P. aquilinum*
L-malic 	+	+	+	+	+	+
D-malic 	—	—	—	—	—	+
D-tartaric 	o	+	o	o	—	o
Mesotartaric 	+	+	+	+	+	+
Racemic 	o	+	o	o	—	—
Succinic 	o	+	o	o	—	o

TABLE 9 (*continued*)

Acid	*E. arvense*	*I. japonica*	*S. natans*	*O. javanica*	*P. sulphurea*	*P. aquilinum*
Monobromosuccinic 	o	+	+	+	+	−
Dibromosuccinic 	o	+	o	o	−	−
Isodibromosuccinic 	o	+	o	o	o	−
Fumaric 	o	+	o	o	o	o
Maleic 	o	o	+	+	+	+
Dihydroxyfumaric * 	−	−	−	−	−	o
Mesaconic 	o	+	o	o	o	o
Citraconic 	o	o	+	+	−	+
Itaconic 	o	+	o	o	−	−

* Until 1953, a bottle labelled dihydroxymaleic acid, which probably would attract some plant spermatozoa, actually contained dihydroxyfumaric acid (Hartree, 1953), which does not attract.

TABLE 9 (*continued*)

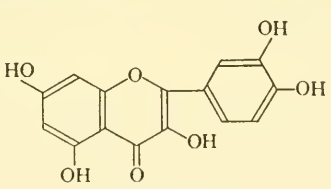
Acid	*E. arvense*	*I. japonica*	*S. natans*	*O. javanica*	*P. sulphurea*	*P. aquilinum*
1,1,1-Ethane-tricarboxylic 	−	+	o	−	−	−
Agaricic 	o	+	o	−	−	−
Tartronic 	−	−	−	−	−	+
Suberic 	o	+	o	o	−	−
Sebacic 	o	+	o	o	−	−
Glutaric 	o	+	o	−	−	−
cis-Camphoric 	o	o	o	o	−	−
trans-Camphoric 	o	+	o	−	−	−

Notes: The following acids are inactive: aconitic, oxalic, malonic, formic, glycollic, lactic, acetic, saccharic, mucic, aspartic, glutamic, phthallic, terephthallic and α-truxillic acids. Citric acid was reported by Bruchmann (1909) to attract the spermatozoa of *Lycopodium clavatum* Linn., but it has no effect on the spermatozoa of the plants mentioned in this table.

CHAPTER 4

SPERM-EGG INTERACTING SUBSTANCES, III

THE shrubs of *Forsythia* × *intermedia* Zabel have two sorts of flowers, which are not found growing together on the same bush. In one the styles are short and the stamens long, while in the other, the styles are long and the stamens short (Plate V). In nature, fertilization only occurs when pollen from a flower with long stamens comes into contact with a stigma on a long style (on another flower), or when pollen from a flower with short stamens comes into contact with a stigma on a short style (on another flower). In addition, and for reasons which are obvious from what has been said, *Forsythia* is self-sterile. Not only does the pollen from one flower never fertilize the same flower (self-sterility); it also never fertilizes a flower of the same type whether on the same bush or another. Moewus (1949, 1950) claimed to have found that when water extracts of *Forsythia* pollen and stigmata were mixed, the flavonol, quercetin, was found in some cases and not in others. Table 10 shows which combinations of pollen and stigmata produce



Quercetin

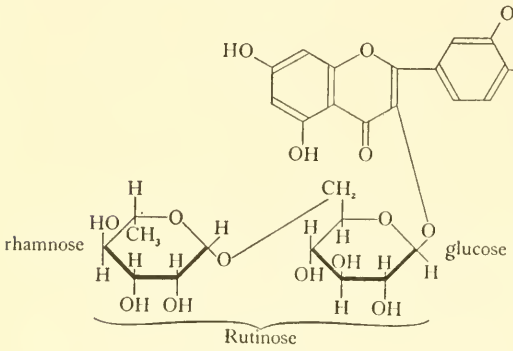
quercetin and which do not. As the amounts of quercetin formed were small and could not be identified by chemical analysis, Moewus used a biological method of identification, which, being of considerable gametological interest, will be described later.

Further investigations by Kuhn & Löw (1949*a*) appear to have revealed that pollen from long stamens contained rutin, while that obtained from short stamens contained quercitrin, the yields of these substances from pollen being about 10% in each case. Rutin is found in the petals of both types of flower (Kuhn & Löw, 1949*b*), in approximately equal quantities; it is only in the pollen

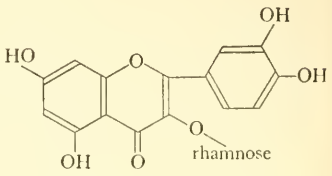
TABLE 10

Stigma from	*Pollen from*	*Quercetin*
Long style	Long stamen	Present
Short style	Short stamen	Present
Long style	Short stamen	Absent
Short style	Long stamen	Absent

that the different flavonol glycosides are found. These observations, if they are correct, are important in that, for the first time, self-sterility and the specificity of fertilization can be related to



Rutin (long stamens)



Quercitrin (short stamens)

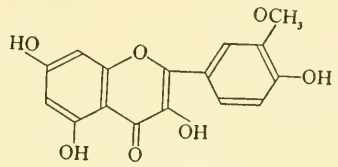
specific chemicals occurring in the sexual organs. The production of quercetin, the aglucone of rutin or quercitrin, following the interaction of the 'right' sort of pollen with the 'right' sort of stigma, is achieved by hydrolysing enzymes, presumably acting in a similar way to rhamnodiastase, which is obtained from Chinese buckthorn. These hydrolysing enzymes are said to be located in a thin layer of cells covering the top of the stigma. The two types of enzyme are specific, the one in 'short' stigmata hydrolysing quercitrin to quercetin, while that in 'long' stigmata hydrolyses rutin to quercetin.

Moewus' biological identification of quercetin has been mentioned. Three clones of the alga *Chlamydomonas eugametos* Moewus are used: Clone 1, which is obtained by subjecting female cells to temperature shock, consists of mutants which, unlike the original organisms, do not produce *iso*-rhamnetin. The inability of these

PLATE V

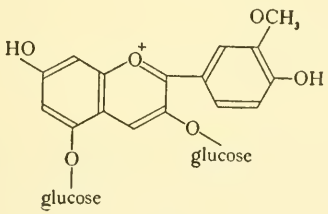
The two sorts of flower of *Forsythia* × *intermedia*. Left, long styles and short stamens; right, short styles and long stamens. Photograph by Royal Botanic Gardens, Kew.

mutants to produce *iso*-rhamnetin is due to their inability to

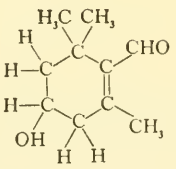


iso-Rhamnetin

synthesize the intracellular precursor of this substance, quercetin. In the presence of added quercetin they can produce *iso*-rhamnetin and then become able to copulate with male gametes. Clone 2 is a homothallic strain which normally produces female *and* male gametes. When Clone 2 cells are treated with peonin or 4-hydroxy-β-cyclocitral, which induce male characteristics, all of them copulate with female cells if these are available; if, however, homothallic cells are treated with *iso*-rhamnetin, which induces female characteristics, all of them pair with male cells. Clone 3 consists of normal male cells.



Peonin



4-hydroxy-β-cyclocitral

When a solution is believed to contain quercetin, it is added to a suspension of Clone 1; if quercetin is present, the Clone 1 cells produce *iso*-rhamnetin. Clone 2 cells are then treated with the solution containing *iso*-rhamnetin. This causes them to react as female cells so that Clone 3 cells will copulate with them. The test procedure can therefore be summarised as follows:

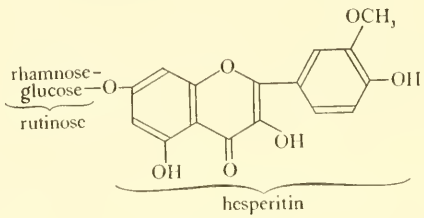
Test solution $\longrightarrow$ Clone 1 $\longrightarrow$ Clone 2 $\longleftarrow$ Clone 3

If Clone 3 cells copulate with Clone 2 cells, quercetin is present in the test solution.

As often happens, other scientists have been unable to confirm Moewus' findings. Lewis (1954, p. 266), for example,

says that 'T. O. Dayton, working in this laboratory, has made chemical tests on pollen of a number of different plants from two species, including *Forsythia intermedia* var. *spectabilis*. In none of the pollen from either pin or thrum plants could quercitrin be detected, and all contained rutin. I have tested the effect of boric acid on pollen-tube growth * on incompatible stigmas but with negative results.' Although Lewis suggests a possible explanation of these negative results, the grave doubts which have been expressed about Moewus' *Chlamydomonas* work (see for example Förster & Wiese, 1954), coupled with these recent findings on *Forsythia*, must leave all scientists with very uncomfortable feelings.

Apart from the work of Moewus & Kuhn on the role of flavanone derivatives in plant gametology, claims have been made that



Hesperidin

phosphorylated hesperidin, another flavanone derivative, inhibits fertilization in mammals. Work published on this subject does not make clear at what points in the hesperidin molecule phosphorylation occurs. In 1952 Martin & Beiler reported that oral or intraperitoneal administration of phosphorylated hesperidin inhibited conception in 44 out of 54 rats. The substance was said to exert its effect on the female and not the male rats. Previously, these two workers had found that phosphorylated hesperidin inhibited the enzyme hyaluronidase and it was this fact which prompted them to investigate its effect on conception in rats. Later in 1952, Sieve claimed that out of 300 married couples he had persuaded to take phosphorylated hesperidin (orally) as a possible contraceptive measure, only two had conceived during the experimental period and that these two couples were unreliable. Unlike Martin

* Moewus (1950) stated that quercitrin and rutin at a dilution of $1 : 10^6$ were inhibitors of pollen germination in sugar solution and that this inhibition was counteracted by 0.01% boric acid.

& Beiler in their rat experiments, Sieve said it was essential for the men to take phosphorylated hesperidin, as well as the women. Sieve's claims should not be accepted until they have been independently confirmed, but this may be difficult, as in his paper he does not reveal the structure of the phosphorylated hesperidin used in his experiments, though he says that only one of the possible phosphorylated compounds is efficacious. More recently Chang & Pincus (1953) have repeated and failed to confirm Martin & Beiler's results. They say (p. 275) that 'phosphorylated hesperidin does not inhibit fertilization when deposited into the Fallopian tubes of rabbits at the time of sperm penetration, nor does it inhibit ovulation, implantation, or normal development of the embryo when administered intraperitoneally or orally to rats.' In a 1% solution, phosphorylated hesperidin did, however, impair the fertilizing capacity of rabbit spermatozoa, but, as Chang & Pincus point out, such a high concentration would be unlikely to be achieved after oral or intraperitoneal administration.

CHAPTER 5

THE METABOLISM OF EGGS, I

Oxygen uptake. In 1908 Warburg did some famous experiments showing that fertilization caused a sharp and immediate increase in the respiration of sea-urchin eggs. The results of a more recent, manometric experiment on this subject, with KOH in the centre well of the flask, are given in Fig. 9. The difference between the pre- and post-fertilization rates of O_2 uptake is about 600%. During the first five minutes after the addition of spermatozoa to the egg suspension, respiration appears to have completely stopped; but this is an illusion caused by the transient production of egg acid which takes place at fertilization (q.v.). This acid displaces CO_2 from the bicarbonate in the sea water at such a rate that the KOH in the centre well cannot absorb it immediately. As a result, the negative pressure in the manometer, due to the disappearance of oxygen, is temporarily masked by the positive pressure of the evolved CO_2.

In spite of the experiments of Loeb & Wasteneys (1913) on starfish eggs, in which no comparable rise in respiration after fertilization was found, the belief used to be widely held that fertilization was associated with an increase in oxidative activity, and that since an embryo would have to do more work, it would obviously require more oxygen than an inert, unfertilized egg. As we shall see, this led to further attractive but untenable ideas, for example that 'cytochrome' is thrown into circulation at fertilization. A new light was shed on the famous phenomenon of increased O_2 uptake at fertilization when Whitaker (1931*a*, *b*) pointed out that though O_2 uptake increased when the eggs of *Arbacia punctulata*, *Fucus vesiculosus* and *Nereis succinea* were fertilized, there was a negligible increase in the case of *Sabellaria alveolata* (Linn.), and a decrease in the eggs of *Cumingia tellinoides* and *Chaetopterus variopedatus* (Fig. 10). Nor is there any increase in O_2 uptake when the eggs of *Saxostraea commercialis* (Iredale & Roughley) are fertilized (Cleland, 1950*a*), in the frog's egg (Brachet, 1934*b*), nor in some batches of eggs of *Urechis caupo* (Tyler & Humason, 1937). The next discovery of interest in this

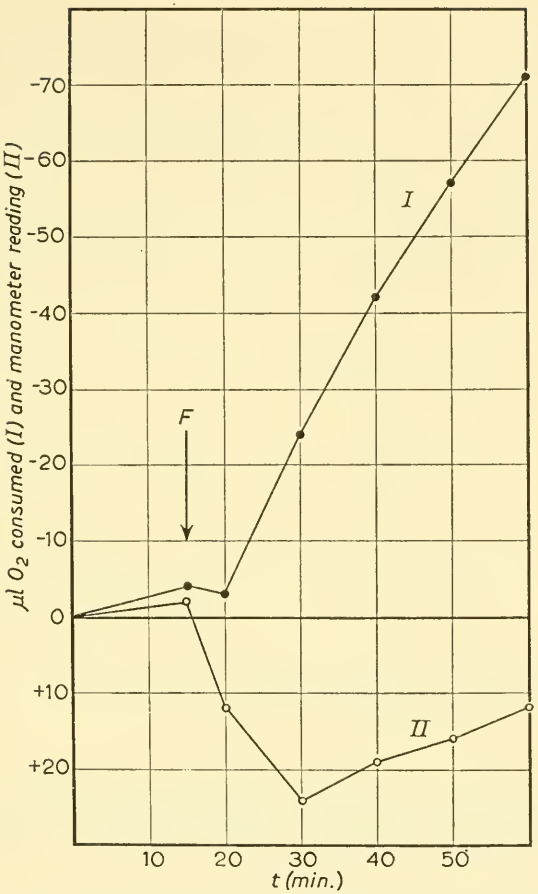


FIG. 9.—Metabolism of eggs of *Paracentrotus lividus*, before and after fertilization. *F*, addition of spermatozoa. Gas phase, air. *I*, with 0·2 ml. 10% KOH in the centre well of the manometer flask; *II*, without KOH. Both curves corrected for sperm respiration. T° C., 20.

field was due to Holter & Zeuthen (1944), who found that the respiration of unfertilized eggs of *Ciona intestinalis* (Linn.) declined with time after removal from the female. A few years later, Borei (1948, 1949), using the Cartesian diver technique, examined the variation in the O_2 uptake of unfertilized sea-urchin eggs (*Psammechinus miliaris*) with time after removal from the ovary. His results are shown in Fig. 11. If it were possible to measure the respiration of unfertilized sea-urchin eggs immediately after

shedding and to fertilize them at once, it seems probable that no increase in O_2 uptake would be observed after fertilization. In fact, reference to Fig. 11 shows that there might well be a decline in O_2 uptake, though at a later stage in embryonic development there would, of course, be the well-known increase in respiration. In nature, sea-urchin eggs are fertilized soon after shedding, for reasons which are gone into at length in chapter 2, Sperm-Egg Interacting Substances, I. Claims that cytochrome is 'thrown into circulation' at fertilization, or that, before fertilization, there is limited contact between respiratory enzymes such as cytochrome

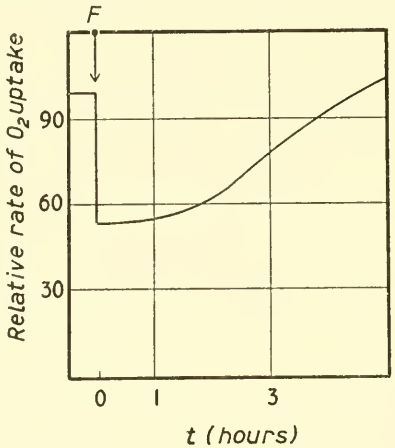


FIG. 10.—Rate of O_2 uptake of eggs of *Chaetopterus variopedatus*. *F*, addition of spermatozoa. T° C., 21. The post-fertilization rate is expressed as a percentage of the pre-fertilization rate (100), which is -24 $\mu l O_2$/hour/10 μl eggs. After Whitaker (1933*a*).

and substrates in the egg (Runnström, 1930); or that there is a block in the chain of carriers at this time (Runnström, 1935*a*); or that unfertilized eggs are in a biochemically similar condition to insect embryos in diapause (Needham, 1942), lose some of their value in the light of Borei's experiments. It is, of course, interesting to know that unfertilized eggs preserve their substrates by a reduction in metabolic activity when allowed to remain unfertilized for hours rather than minutes, a frequent concomitant of their preparation for manometric experiments; but generalisations and interpretations based on a study of biological material in abnormal conditions are apt to be misleading, particularly if the abnormal features have escaped the notice of the experimenter.

Cleland (1950*a*) was unable to observe any decline in O_2 uptake with time after removal from the ovary, in unfertilized oyster eggs, in Warburg manometers. But sooner or later, being shaken in manometers has an injurious effect on unfertilized eggs, which is reflected in pathological increases in O_2 uptake. Alternatively, the apparent increases in the O_2 uptake of unfertilized eggs may be

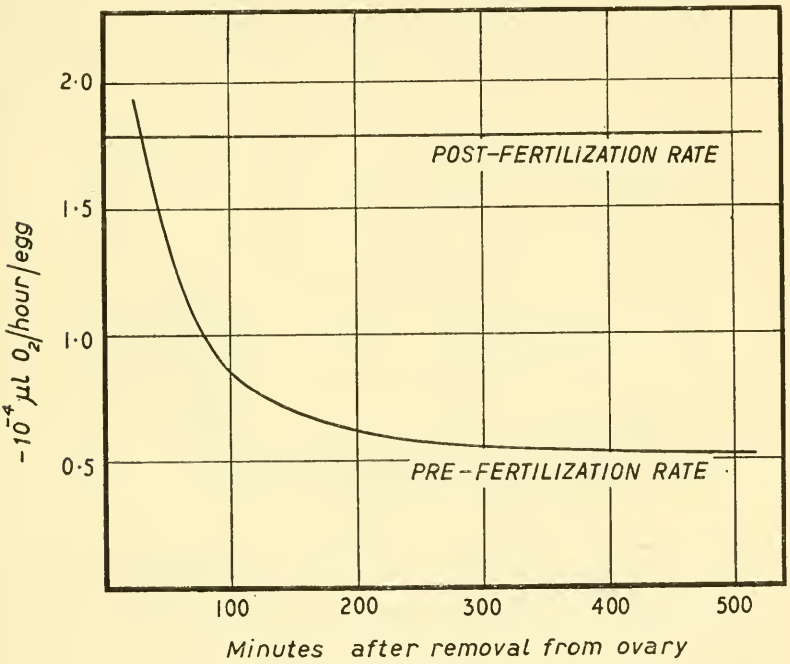


FIG. 11.—Comparison of pre- and post-fertilization O_2 uptake of eggs of *Psammechinus miliaris*, after Borei (1949). The asymptotic O_2 uptake of the unfertilized eggs, $5 \cdot 10^{-5}$ µlO_2/hour/egg, is only about $\frac{1}{7}$th of the O_2 uptake in Fig. 13.

due to the growth of bacteria in the suspension, though, admittedly, it takes some while for this effect to become significant. Tyler *et al.* (1938) carried out some interesting experiments, Fig. 12, comparing the respiration of unfertilized eggs of *Arbacia punctulata* in sterile and non-sterile media. The experiments show that contamination of cultures with bacteria and the metabolism of the latter are factors which require careful attention in experiments of this sort. Some of these factors may explain Cleland's results; but

Borei's findings are of sufficient importance to warrant their being repeated and it is to be hoped that this will soon be done.

We must now enquire into the reasons for the different respiratory responses of eggs to fertilization. Three explanations have been put forward, involving respectively the state of maturation of the egg at fertilization, the pre-fertilization level of O_2 uptake, and changes in the nature of unfertilized egg metabolism, according to their and their parents' history. Reference was made in chapter 1 to the four different states of egg maturation at which fertilization occurs. Table 11 gives some details of the ratio (O_2

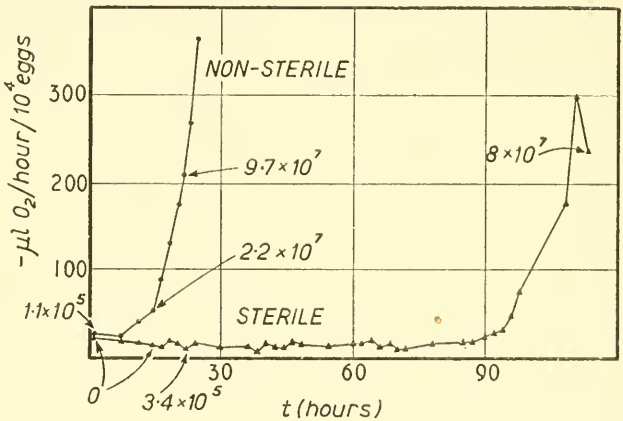


FIG. 12.—O_2 uptake of unfertilized eggs of *Arbacia punctulata* under sterile and non-sterile conditions. The figures against each curve refer to the number of bacteria per ml. of egg suspension. After Tyler *et al.* (1938).

uptake, fertilized eggs)/(O_2 uptake, unfertilized eggs) in each of the four classes. Further information will be found in papers by Ballentine (1940) and Cleland (1950*a*). The ratio will be seen to be about 1, or less, in Classes 2 and 3, not in general much more than 1 in Class 1, and markedly more than 1 only in Class 4. There are a few exceptions; but the different temperatures at which the experiments were done and the variations in time after removal from the ovary, may be responsible for these. The table shows, however, that when the nucleus is in what is sometimes called the 'kinetic' state, there is no increase in O_2 uptake at fertilization, but that when it is quiescent, O_2 uptake increases at fertilization. The increase may, in fact, be caused by sperm penetration or the onset of meiosis. The Class 2 situation, in which the ratio may be less

than 1, suggests that the increased respiration at the onset of the meiotic divisions is not merely what might be expected in a dividing cell, but that breakdown of the germinal vesicle is the responsible factor. This latter point is confirmed by the experiments

TABLE 11

The ratio (O_2 uptake, fertilized eggs)/(O_2 uptake, unfertilized eggs) in various organisms

	$-O_{2f}/-O_{2u}$	
Class 1		
f. at germinal vesicle stage		
N. succinea	1·3	Barron, 1932
M. laterialis	1·8	Ballentine, 1940
U. caupo	1·2	Tyler & Humason, 1937
Class 2		
f. at first maturation metaphase		
C. intestinalis	1	Holter & Zeuthen, 1944
C. tellinoides	0·45	Whitaker, 1931*b*
S. commercialis	1	Cleland, 1950*a*
C. variopedatus	0·53	Whitaker, 1933*a*
M. glacialis	1	Borei, 1948
S. alveolata	1·1	Fauré-Frémiet, 1922
C. intestinalis	1·5	Tyler & Humason, 1937
Class 3		
f. at second maturation metaphase		
R. temporaria	1	Zeuthen, 1944
B. bufo	1	Stefanelli, 1938
F. heteroclitus	16·7	Boyd, 1928
F. heteroclitus	1	Philips, 1940
Class 4		
f. after maturation		
F. vesiculosus	1·9	Whitaker, 1931*a*
S. purpuratus	3·7	Tyler & Humason, 1937
P. miliaris	3·6	Borei, 1948
P. lividus	4·7	Brock *et al.*, 1938
A. punctulata	4·5	Ballentine, 1940

of Borei (1948) on the eggs of *Marthasterias glacialis* (Linn.) in which respiration increases during the breakdown of the germinal vesicle and the meiotic divisions. In the case of the latter, the increase persists *after* the meiotic divisions are completed. Cleland (1950*a*) also found a difference in the respiration of oyster eggs, before and after the breakdown of the germinal vesicle.

The weird case of the eggs of *Fundulus heteroclitus* (Linn.) requires re-investigation, particularly since Nakano (1953) found no changes in O_2 uptake after fertilization of the eggs of *Oryzias latipes* (Temminck & Schlegel). Very little respiratory increase occurs in this egg until about two hours after fertilization.

Whitaker (1933*b*) has put forward the view that the post-fertilization level of respiration, and therefore the change in respiration at fertilization, is dependent on the pre-fertilization level, as many fertilized eggs tend to have the same respiratory rate, $-(1\text{–}2)\mu l O_2$/hour/10μl eggs at 21° C, while the pre-fertilization rate may be well above or below this level. Whitaker has adduced convincing evidence in favour of this contention, which Brachet supports in *Chemical Embryology*. One might summarise Whitaker's hypothesis by saying that the respiration of the unfertilized egg is *regulated* by fertilization, many eggs which, before fertilization, have widely different rates of O_2 uptake, approaching the same rate after fertilization.

The idea that the past history of eggs and the females from which they were obtained may influence the respiratory response to fertilization was first put forward by Tyler & Humason (1937), following their experiments on the eggs of *Urechis caupo*. Fertilization of these eggs may produce an increase, a decrease, or no change in O_2 uptake, depending on how long the animals have been kept in the aquarium tanks. When eggs are obtained from a female which has not been kept long in the aquarium, the unfertilized rate is relatively high; but in eggs from a female which has been in the aquarium for a considerable time, and which is therefore starved, the unfertilized rate is low. Kavanau (1954*b*), in his studies on the amino acid metabolism of sea-urchin eggs, claims that the high respiratory rate of eggs freshly removed from the ovary, and of oocytes, is due to the high energy requirements of yolk protein synthesis, which he believes goes on at this time. Kavanau's results are discussed in more detail in the next chapter, but it seems doubtful whether this can be the whole explanation of the wide variation in respiration observed in eggs of different species. It is, however, clear that in future, more attention must be paid to the history both of the eggs and of the females from which they were obtained, before the experimental period.

When the rate curves immediately after fertilization are examined, Fig. 13, it will be observed that, apart from any steady

increase in the level of O_2 uptake, fertilization is followed by a transient increase in respiration, even though rate curves tend to over-emphasize such effects. According to Boyd (1928), the same thing happens in the eggs of *Fundulus heteroclitus*, though the time

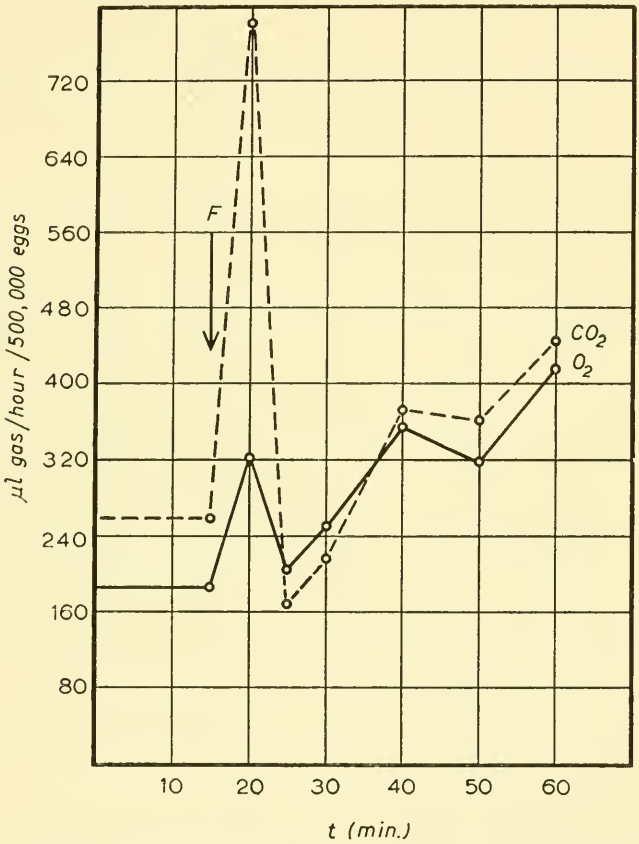


FIG. 13.—Total CO_2 production and O_2 uptake in μl gas/hour, before and after fertilization of eggs of *Psammechinus miliaris*. *F*, addition of spermatozoa. Warburg indirect method. Gas phase, 0·3% CO_2 in O_2. T° C., 20. (Laser & Rothschild, 1939).

scale is longer. Whether this always occurs and what its significance is are questions which require further investigation.

Respiratory quotient. After straightforward measurements of O_2 uptake, the next stage in a metabolic investigation often takes the form of an examination of the respiratory quotient (R.Q.), i.e.

(CO_2 produced)/(O_2 consumed). Such measurements are technically rather difficult to do immediately after fertilization, when sea-urchin eggs are the biological material, for the following reasons: first, the primitive method of measuring R.Q. by having two manometric flasks containing eggs and absorbing the evolved CO_2 in one of them (which gives $-O_2$), but not absorbing the evolved CO_2 in the other flask (which gives $+CO_2-O_2$), does not work; because sea-urchin eggs, unlike those of the oyster (Cleland, 1950*a*), respire at higher rates when the bicarbonate content of the sea water is normal than when it is low, following the absorption of CO_2 from the gas phase. Secondly, CO_2 retention must be measured. This is troublesome and necessitates the use of mixtures containing known tensions of CO_2 in the gas phase. Thirdly, unfertilized sea-urchin eggs which have been prepared for manometric experiments and may, therefore, have been out of their ovaries for more than an hour, have a low rate of metabolism, as can be seen from Figs. 9, 11 and 13, and are easily damaged by being shaken in manometers. These difficulties in the systematic repetition of experiments on unfertilized eggs may drive the experimenter to the dangerous expedient of selecting experiments in which 'everything went well'. The apparent R.Q. of unfertilized sea-urchin eggs is about 1·4 (Laser & Rothschild, 1939); earlier workers, such as Ashbel (1929) and Borei (1933) obtained somewhat lower figures, 1·1 and 1–1·2. These experiments only tell one that the ratio (CO_2 produced)/(O_2 consumed) is greater than unity in unfertilized eggs. They provide no information about the nature of the endogenous substrate being metabolized, because the experiments do not enable any distinction to be made between respiratory CO_2 and CO_2 displaced from the sea water by acid diffusing out of the eggs. An overall R.Q. of more than 1 very probably indicates that, in these circumstances, acid is being produced by the eggs; but in the absence of information as to how much, we cannot tell what the true R.Q. is, and, therefore, what substrates are being utilised. The delicacy of unfertilized sea-urchin eggs has already been mentioned. The possibility that shaking them in manometers induces a pathological formation of acid requires further investigation.

In rock-oyster eggs, the R.Q. is 0·8 before fertilization, which is interpreted by Cleland (1950*a*) as being due to the eggs metabolising a mixture of carbohydrates and lipids. The R.Q. is also lower

than 1, 0·69–0·89, in the unfertilized eggs of *Urechis caupo* (Horowitz, 1940); but Brachet (1934*a*) obtained the value 0·99 for the unfertilized frog's egg. These results certainly do not suggest a common endogenous substrate in unfertilized eggs of different species.

Intuitively, one feels that profound changes in metabolism should occur at fertilization and that these may be reflected in the R.Q. of the egg. In the period 5–20 minutes after fertilization of the sea-urchin egg, the R.Q. is 0·66, but in the first 2–10 minutes, even lower values are obtained. The average R.Q. for the first 30 minutes after fertilization is 0·84. These results were obtained by Laser & Rothschild (1939) using the Warburg indirect method. Low values for sea-urchin eggs at somewhat longer times after fertilization have been obtained by Ephrussi (1933), Borei (1933) and Öhman (1940), while Brachet (1950) has reported that the R.Q. of the frog egg falls from 0·99 to 0·66 after fertilization; these latter measurements were done over a very long period, 10–15 hours, and are not, therefore, strictly relevant to the issue of what metabolic changes occur *at* fertilization. Cleland (1950*a*) found no change in the low R.Q. of rock-oyster eggs at fertilization, but Horowitz (1940) states that the low R.Q. which is characteristic of unfertilized *Urechis* eggs disappears at fertilization, and that during the first two hours of development, the R.Q. is 1. These results are probably not so confusing as they superficially seem to be, when one remembers the different *times* after fertilization that measurements have been made and the difference in the morphology of a fertilized egg, 10, 30 and 120 minutes after fertilization. In this book we are only concerned with the early phases of reproduction, the first 60 or so minutes of the egg's existence following fertilization. Metabolism after the fusion of the pronuclei and energy sources during development (Needham, 1942; Brachet, 1950) are not, therefore, considered.

Acid production. The sudden evolution of acid, about 20 μ-moles/100 mg. N, when the sea-urchin egg is fertilized, was first mentioned in 1929 by Ashbel and examined in detail by Runnström (1933). Reference to Fig. 13 shows that the evolution of this acid, or at any rate the bulk of it, is of very short duration, about five minutes. The nature of the acid is unknown; it is neither lactic, pyruvic nor malic acid and, according to Yčas (1950), it is unlikely to be any of the Krebs cycle acids. In spite of Cennamo

& Montella's claim (1947) that the acid formed in cytolysing sea-urchin eggs is phosphoric acid, and the inhibitory action of phlorrhizin on this reaction (Rothschild, 1939), the acid which diffuses out of these eggs at fertilization is not phosphoric acid. Perhaps we should take account of the possibility that no organic acid diffuses out of the egg at fertilization, but that some ion exchange reaction, involving H_3O^+, occurs. According to Cleland (1950*b*, p. 314), who did not observe any acid production when rock-oyster eggs were fertilized, 'experiments on cytolytic acid production . . . suggested beyond reasonable doubt that most of the acid production was non-metabolic.' The word non-metabolic means non-glycolytic; an exchange reaction would presumably come into the 'non-metabolic' category. Alternatively, the reaction

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose-6-phosphate} + \text{H}^+$$

at pH 7, is one suggestive example of a means of producing acid in the form of hydrogen ions.*

It is a remarkable and disappointing fact that acid production at fertilization has not been observed, assuming that anyone except Cleland has looked for it, in eggs other than those of the sea-urchin.

The cytochrome system. After measurements of O_2 uptake and R.Q., the next step might well be to investigate the activity of the cytochrome system in fertilized and unfertilized eggs, as cytochrome oxidase is the terminal enzyme in aerobic catabolism. We can be virtually certain that cytochrome will be present, because it has been found in all aerobic organisms which have so far been examined; but confusion about the existence of cytochrome in sea-urchin eggs and its role, if any, in fertilization, has arisen for two reasons. First, because many workers have failed to observe cytochrome spectroscopically in sea-urchin eggs; it was not until these eggs were examined spectroscopically at the temperature of liquid air (Rothschild, 1949*a*), a technique which

* When considering increases in O_2 uptake at fertilization and reactions involving ATP, the student should note that if, at fertilization, ATP is broken down with liberation of ADP, oxidations may be facilitated, as reactions of the type

$$AH_2 + B \longrightarrow A + BH_2$$

very often cannot take place, unless they occur in the following way

$$AH_2 + B + ADP + P \longrightarrow A + BH_2 + ATP$$

Keilin & Hartree discovered in 1939 and which markedly intensifies the various bands of cytochrome, that cytochrome was clearly seen. The sea-urchin egg spectrum is atypical, as although band *a* is present, the normal bands of *b* and *c* are replaced by one which can be called b_1. Yčas (1954) confirmed these observations. The band of CO-cytochrome oxidase, or COa_3, is too faint to be seen with certainty, though cytochrome oxidase has been identified by Krahl *et al.* (1941). The apparent absence of cytochrome *c*—according to Borei (1951), sea-urchin eggs must contain less than 5×10^{-4} γ/mg. dry matter—is surprising. But in view of the reactions of fertilized and unfertilized eggs to inhibitors of cytochrome oxidase and the increased O_2 uptake observed in the presence of dimethyl-*p*-phenylenediamine (Runnström, 1930), we can be certain that cytochrome *c*, or a carrier which is functionally indistinguishable from it, is present in the sea-urchin egg. Cytochrome *c* is present in the eggs of the rock-oyster (Cleland, 1950*b*). According to Horowitz & Baumberger (1941), the eggs of *Urechis caupo* do not contain cytochrome but an oxidizable and reducible haem pigment, Urechrome, believed to act in a similar way to cytochrome. In spite of this observation, a further examination of these eggs at the temperature of liquid air should be rewarding.

The second reason for the confusion about cytochrome in sea-urchin eggs concerned the inability of early workers to recognise that cyanide, which inhibits the reduction of ferri-a_3, and CO, which in the dark or green light inhibits the oxidation of ferro-a_3, inhibit the respiration of unfertilized eggs. These early failures were for the time being partly responsible for the seductive but false idea that the cytochrome system does not function in the unfertilized egg but is 'thrown into circulation' following fertilization or parthenogenetic activation. It is now known, through the work of Robbie (1946) and Rothschild (1949*a*), that both cyanide and CO reversibly inhibit the respiration of unfertilized sea-urchin eggs. These observations prove that the cytochrome system is present and functioning in these eggs. One of the difficulties encountered in the use of these inhibitors is that, at certain concentrations but not at others, both of them increase the O_2 uptake of unfertilized eggs, as Lindahl observed in 1940. The mechanism of this effect is not clear in the case of cyanide. In the case of CO, the eggs may oxidize CO as in heart muscle (Fenn & Cobb, 1932),

though Lindahl (1938) does not think this likely; alternatively, their respiration may be stimulated by CO, which is known to occur in other tissues (Daly, 1954). A further complicating factor in CO-inhibition studies is that the respiration of unfertilized sea-urchin eggs is depressed in the presence of strong light, which must be used to obtain photo-reversal of CO-inhibition.

In the light of what has been said above, I am inclined to think that, for historical reasons, Needham placed too much emphasis in *Biochemistry and Morphogenesis* on the alleged change-over at fertilization from a non-ferrous to a ferrous type of respiration. In other respects his section on Respiration, pp. 562–605, is excellent and contains a good deal of information omitted, for reasons of space, from this chapter.

To sum up, changes in O_2 uptake at fertilization depend on the time after shedding or removal from the ovary when fertilization occurs; on the state of maturation of the egg at fertilization; and on the previous history of the animals from which the eggs were obtained. These factors may result in there being an increase, no increase, or a fall in respiration at fertilization. The transient increase in O_2 uptake, which occurs immediately after fertilization, has only been observed in sea-urchin eggs (apart from an early experiment, which should be repeated, on *Fundulus* eggs); the same applies to acid production and the low R.Q. at this time. The lack of systematic examination of other eggs, particularly in regard to acid production, is a serious lacuna in our knowledge. The cytochrome system is present and functioning in unfertilized and fertilized eggs. No qualitative changes in the behaviour of this system occur at fertilization.

CHAPTER 6

THE METABOLISM OF EGGS, II

Carbohydrate metabolism. One of the objects of studying the metabolism of eggs is to translate the observed biological results of fertilization into quantitative chemical language. This will probably not be achieved in the next twenty-five years and, as always happens when subjects are in their infancy, our knowledge of the metabolic changes induced by fertilization is disordered and contradictory. We can follow up the investigations described in the previous chapter by asking the following questions: what are the principal sources of energy in the egg and what are the metabolic changes which make this energy available for such purposes as maintenance of structure, pumping sodium out of the egg if that is necessary, rotation of the sperm head, cytoplasmic movements when they occur, aster formation, and movements of the pronuclei? The R.Q. experiments already discussed show that it is far from certain that carbohydrate is always the principal endogenous substrate of eggs; but because our knowledge of the pathways of carbohydrate catabolism is greater than that of lipid or protein catabolism, more attention has naturally been paid to the former than the latter. Tables 12 and 13 summarise the evidence that sea-urchin eggs have a normal anaerobic carbohydrate metabolism, or at any rate the machinery for it. The reason for the qualification is that, because of the impermeability of eggs (unlike spermatozoa, Mann, 1951), experiments to investigate metabolic pathways in eggs almost always necessitate the use of homogenates, with or without the addition of various cofactors; in these experimental conditions, the normal egg structure and, therefore, conceivably, the normal egg metabolism, is destroyed.

Cleland (1950*a*, *b*) has obtained evidence which is very similar to that given in Tables 12 and 13 for the existence of a classical glycolytic system in the eggs of the rock-oyster.

In spite of what has been said above, opinions are not unanimous about the principal pathways of carbohydrate metabolism in the sea-urchin egg. Lindberg & Ernster (1948) were the first to suggest that the mechanism often known as the hexose monophosphate

TABLE 12

Evidence for the existence of a classical glycolytic cycle in sea-urchin eggs

Lower case, not identified; clarendon upper case, identified, or claim for existence strong; upper case, claimed to exist.

	'GLYCOGEN' [1, 2, 3]	
Hexose —(HEXOKINASE [6, 7]; ATP [8, 9])→	Glucose-1-phosphate	PHOSPHORYLASE [3]
	Glucose-6-phosphate	PHOSPHOGLUCOMUTASE [3, 4, 5]
	Fructose-6-phosphate	PHOSPHOHEXOISOMERASE [3, 4, 6]
	Fructose-1, 6-diphosphate	PHOSPHOFRUCTOKINASE [5]
	DIHYDROXYACETONE PHOSPHATE } [4] D-GLYCERALDEHYDE-3-PHOSPHATE }	**ALDOLASE** [3, 4, 5]
	D-1,3-diphosphoglyceric acid	TRIOSE PHOSPHATE DEHYDROGENASE [3, 4, 5]
	D-3-phosphoglyceric acid	Transphosphorylase
	D-2-phosphoglyceric acid	Phosphoglyceromutase
	PHOSPHOENOLPYRUVIC ACID	**ENOLASE** [3, 4]
	PYRUVIC ACID [3]	Transphosphorylase
	LACTIC ACID [3, 4, 10, 11, 12]	LACTIC DEHYDROGENASE, [3, 4] **DPN** [13]

1. Örström & Lindberg, 1940.
2. Hutchens *et al.*, 1942.
3. Cleland & Rothschild, 1952*a*.
4. Yčas, 1954.
5. Krahl *et al.*, 1954*a*.
6. Krahl *et al.*, 1953.
7. Krahl *et al.*, 1954*b*.
8. Connors & Scheer, 1947.
9. White & Chambers, 1949.
10. Perlzweig & Barron, 1928.
11. Zielinski, 1939.
12. Runnström, 1933.
13. Jandorf & Krahl, 1942.

TABLE 13

Further evidence for the existence of a classical glycolytic cycle in sea-urchin eggs (*Cleland & Rothschild, 1952b*)

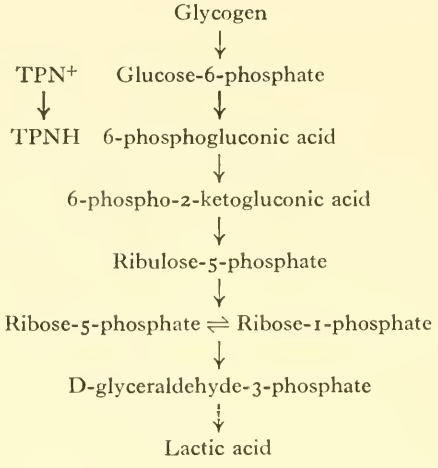
1. Accumulation of pyruvate and lactate anaerobically and in presence of HCN.
2. Inhibition of O_2 uptake by fluoride and reversal by pyruvate.
3. Accumulation of pyruvate after addition of glucose-6-phosphate.
4. No accumulation of pyruvate after addition of phosphogluconate.
5. Active oxidation of hexose diphosphate with pyruvate accumulation.
6. Stimulation of endogenous O_2 uptake by DPN.
7. Stimulation of fructose oxidation by DPN.
8. Inhibition of 6 and 7 by fluoride and iodoacetate.

shunt, or an oxidative pathway, was mainly responsible for carbohydrate breakdown in sea-urchin eggs. As these pathways may not be so familiar to some readers as the usual glycolytic one, they are reproduced on p. 72.

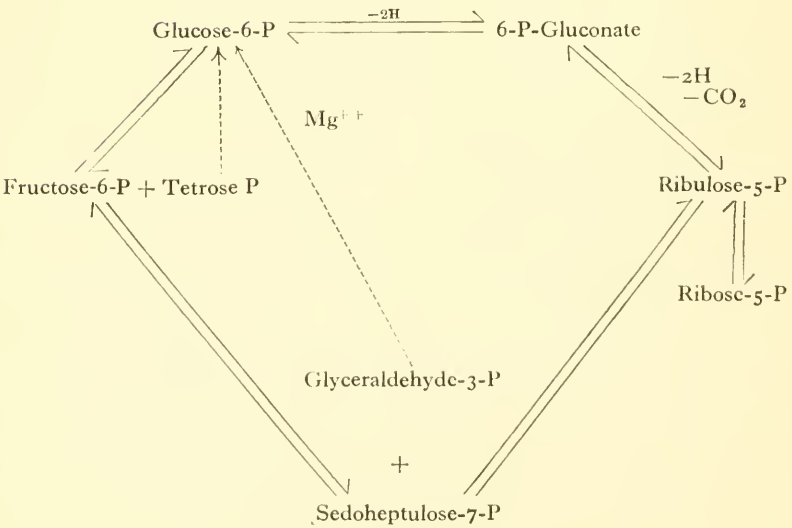
The evidence in favour of the operation of a scheme along these lines in sea-urchin eggs is: (*a*) egg homogenates actively metabolise phosphogluconate. In addition, Krahl *et al.* (1955) have shown that *Arbacia* homogenates contain glucose-6-phosphate and 6-phosphogluconate dehydrogenases, and can form ribose from either substrate. These workers found that the rate of TPN reduction with glucose-6-phosphate as substrate was about 3 μ-moles/min./g. eggs, enough to permit an O_2 uptake six times that of fertilized and twenty-four times that of unfertilized eggs. On the other hand, the rate of DPN reduction by egg extracts with fructose-1, 6-diphosphate as substrate was 0·1–0·2 μ-moles/min./g. eggs. At the same temperature, the O_2 uptake of the fertilized eggs would require 0·5 μ-moles of DPN to be reduced if all the O_2 uptake were associated with the glycolytic breakdown of carbohydrate. Although all the O_2 uptake of fertilized eggs is almost certainly not associated with carbohydrate breakdown, the amount of carbohydrate which can pass the aldolase-oxidizing enzyme step appears to account for only 20–40% of the oxygen actually consumed in the eggs of *Arbacia punctulata*. (*b*) Low concentrations of iodoacetate do not inhibit the endogenous O_2 uptake of egg homogenates; there are differences of opinion on this point. Cleland & Rothschild (1952*a*) found that iodoacetate did inhibit glycolysis in egg homogenates of *Echinus esculentus*, and so did Yčas (1954), using egg homogenates of *Strongylocentrotus purpuratus* and *Lytechinus pictus*. Lindberg & Ernster's experiments were done in

TABLE 14

A, *hexose monophosphate shunt*; **B**, *oxidative breakdown of carbohydrate, after Horecker* (1953). *One mole of* CO_2 *is evolved, two moles of TPN are reduced, and one mole of* O_2 *is consumed, per cycle. Two complete cycles will regenerate one mole of glucose-6-phosphate and will produce one mole of tetrose phosphate, which, for the completion of the carbohydrate oxidation, must be converted to glucose-6-phosphate.*



A



B

the presence of 0·1M-NaF, which would make it most unlikely that any glycolysis would have been observed. In any case, rather low concentrations of iodoacetate were used and little time was allowed for the development of the inhibition. The hexose monophosphate shunt, Table 14**A**, will, of course, be just as sensitive to iodoacetate as the glycolytic pathway. If, on the other hand, the hexose monophosphate shunt is not involved in the terminal part of the glycolytic pathway, iodoacetate should not have any inhibitory action. (*c*) The addition of hexose monophosphate caused a greater stimulation of O_2 uptake than hexose diphosphate. This observation also is the subject of dispute; (*d*) significant amounts of the phosphorylated intermediates of glycolysis, e.g. alkali-labile phosphate (triose phosphate), could not be identified by Ba fractionation of acid egg extracts; (*e*) triose phosphate dehydrogenase was said to be absent by Jandorf & Krahl (1942). Table 12 shows that this is probably incorrect.

To sum up, there are evidently two pathways by which carbohydrate is broken down in the sea-urchin and oyster egg, the normal anaerobic and also an oxidative pathway; except in the case of *Arbacia* eggs, where the 'strength' of the latter mechanism appears to be greater than that of glycolysis, at any rate in homogenates, we do not know which mechanism predominates, though both probably function to a greater or lesser extent.

Quite apart from being involved in the degradation of polysaccharides to provide phosphate bond energy, the hexose monophosphate reaction may be concerned with entirely different cellular activities. The reaction

Nicotinamide riboside + phosphate $\rightleftharpoons$ Nicotinamide + ribose-1-phosphate,

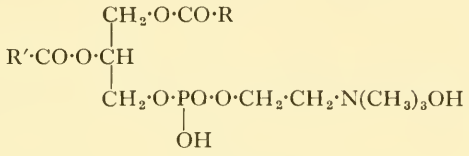
discovered by Rowen & Kornberg (1951), suggests that the hexose monophosphate reaction may be involved in nucleic acid metabolism, on the synthetic and not the catabolic side. Some interesting speculations about the role of this reaction in determination, and, in particular, animalization, will be found in Hultin's review, *Studies on the Structural and Metabolic Background of Fertilization and Development* (1953*a*).

Transient changes in carbohydrate metabolism at fertilization. Zielinski (1939) and later Örström & Lindberg (1940) showed that the 'glycogen' content of sea-urchin eggs decreases at fertilization; in terms of glucose, the breakdown is equivalent to 26 μ-moles/100

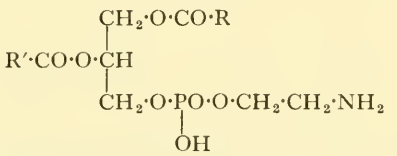
mgN. The effect is a transient one, the rate of 'glycogenolysis' nearly returning to the pre-fertilization level ten minutes after fertilization. In spite of the acid production at fertilization, this breakdown is not associated with the accumulation of lactic acid or any of the tricarboxylic acids; moreover, the amounts of inorganic phosphate, pyrophosphate and hexose phosphates have been said not to change at fertilization (Runnström, 1933; Zielinski, 1939; Örström & Lindberg, 1940), though we shall see in the next chapter that recent experiments, in which more refined methods were used, do not entirely support these findings. If the low R.Q. during the first few minutes after fertilization is linked to carbohydrate metabolism, which seems unlikely, we must conclude that an obscure and transient reaction, involving the incomplete oxidation of carbohydrate and about which, at present, there are practically no clues, must occur, transiently, at fertilization. Both Brachet, in *Chemical Embryology* (1950), and Runnström, in *The Mechanism of Fertilization in Metazoa* (1949), seem to favour the view that the disappearance of polysaccharide at fertilization has something to do with the hexose monophosphate pathway. If this system is only acting as a partial by-pass of normal glycolysis, lactic or pyruvic acid should be formed and oxidized in the usual way. If, on the other hand, the hexose monophosphate reaction is concerned with nucleotide metabolism, why should it only last for ten minutes after fertilization? The lack of answers to these questions indicates, as usual, the need for more experiments.

Lipid metabolism. The low R.Q. at fertilization suggests the transient oxidation of fats at this time, quite apart from the possibility of fats being an important endogenous substrate before fertilization. The most systematic study of this subject is due to Öhman (1945), who found that the total lipid content of the eggs of *Echinocardium cordatum*, which is about 200 mg/100 mg N, decreases for some seven hours after fertilization. Of these lipids, the choline-containing phosphatides or lecithins decrease as development proceeds, while the cephalins or non-choline-containing phosphatides increase. Ten minutes after fertilization, 29% of the free cephalin in the egg becomes bound to proteins. The difference between free and bound cephalin is based on the solubility of the former in ether-chloroform mixtures and the solubility of the latter in chloroform-ethanol mixtures. In an earlier paper, Öhman (1942) reported that, at fertilization, there was a reduction

in free (i.e., extractable with ether-chloroform mixtures) 'cholesterol'; but in his 1945 paper, Öhman says that his previous observation was unreliable and that there is no change in the 'cholesterol' content of the eggs at fertilization. Monroy & Ruffo (1945), on the other hand, reported an increase in free 'cholesterol' at fertilization in sea-urchin eggs. The word cholesterol is placed in quotation marks above, because there is some doubt whether the substance whose concentration was measured by Öhman, Monroy & Ruffo, and earlier workers such as Mathews (1913) and Page (1923), was actually cholesterol or a related steroid.



α-Lecithin



Phosphatidylaminoethanol, a simple cephalin

R·COOH and R′·COOH are fatty acids

Nitrogen metabolism. Important work in this field was done by Örström in 1941. He reported that, at fertilization or parthogenetic activation, there was a transient production of ammonia, lasting for about ten minutes, and that simultaneously, a substance which liberated ammonia upon heating and which Brachet (1950) thinks may be a glutaminylpolypeptide, disappears. Örström believed that the ammonia production resulted from the deamination of nucleic acid. However, this view was based on the deamination of muscle adenosine and adenylic acid by unfertilized egg homogenates, a reaction which he said was accelerated by the addition of cytolysed spermatozoa. Örström also claimed that fertilized eggs differed from unfertilized ones in being able to synthesize glutamine from glutamic acid and ammonia, though both unfertilized and fertilized eggs can effect the reverse reaction, the hydrolysis of glutamine to glutamic acid and ammonia. Apart from transiently producing

ammonia, Örström observed that the NH_3-binding capacity of fertilized sea-urchin eggs was higher than that of unfertilized eggs. This observation has been confirmed by Hultin (1950*c*) who says that the maximum rate of ammonia uptake occurs during the formation of the fertilization membrane. It may seem curious that, in the absence of added ammonia, this is precisely the time at which the eggs are producing the maximum amount of ammonia. When sea-urchin eggs are allowed to develop in the presence of ^{15}N-glycine or ^{15}N-DL-alanine, and subsequently extracted with KCl or sucrose solutions, insoluble proteins, which are probably derived from the microsomes, are found to incorporate the isotope rapidly in the early phases of development, while the soluble proteins do not (Hultin, 1953*a*). Hultin interprets these results as showing that soon after fertilization, there is a 'rebuilding or multiplication of small, cytoplasmic granules, containing ribonucleic acid' (p. 18), a process which is intensified during determination. In spite of the advances made in recent years in accumulating information about nucleotide, protein, and amino acid metabolism in developing sea-urchin eggs, it is unfortunate that Örström's original work has not been systematically repeated and, therefore, remains largely unconfirmed. Even the transient production of ammonia at fertilization has not been observed by other workers and if this experiment could be done fifteen years ago, it could easily be repeated now.

Kavanau (1953, 1954*a*, *b*) has made interesting observations on the amino acid metabolism of sea-urchin eggs. He observed cyclical changes in amino acid concentration (free, peptide bound, and derived from proteins), in fertilized eggs of *Paracentrotus lividus*, but not in the eggs of *Strongylocentrotus purpuratus*; the failure to detect these changes in the latter may have been due to unavoidably poorer experimental conditions. The experiments were done rather a long time after fertilization, from the point of view of this book, though the fall in protein insoluble in a mixture of 0·1M-KCl and 0·011M-acetic acid, in total protein, and in total amino acid concentration, two to three hours after fertilization, is striking. Kavanau believes that an abrupt change in amino acid metabolism occurs at fertilization, yolk synthesis stopping and intense yolk-protein degradation starting, the latter being a major source of energy. In the case of unfertilized eggs Kavanau observed an increase in protein and a decrease

in non-protein amino acids after ageing in sea water. These changes are said to reflect the fact that unfertilized eggs synthesize their own yolk proteins, free amino acids and peptides being supplied to them from other cells in the ovary; though this is not in accord with the fairly prevalent view that large molecules such as proteins are supplied to the growing oocyte in finished form. Unfertilized eggs from freshly collected *Strongylocentrotus purpuratus* had a high free amino acid content which dropped after fertilization, but this was not so in unfertilized eggs obtained from urchins kept under conditions inhibiting spawning. In the latter, the free amino acid content increased after fertilization. Kavanau states that a high free amino acid content is characteristic of eggs in which yolk synthesis is proceeding rapidly, which suggests under-ripeness; a low free amino acid content means that yolk synthesis has ceased, free amino acids and small peptides are no longer being absorbed, and the eggs are ripe (or perhaps even over-ripe), and ready to be spawned. A different interpretation of the facts is, however, possible. The low free amino acid content of unfertilized eggs obtained from animals kept under conditions which inhibited spawning and feeding may be correlated with the nutritional state of the animal, rather than with changes in the synthetic activities of the egg. Freshly collected and, presumably, well fed animals might be expected to be relatively rich in free amino acids, while stored and probably semi-starved animals might have a rather low content of them, the difference being reflected in all the body tissues, including the ovaries and eggs. According to Kavanau (1954*a*, p. 566), 'the early post-fertilization changes in non-protein amino acids (and probably other metabolites) must apparently be viewed as adjustments which bring an initially flexible system to a more rigidly defined state from which normal development can proceed.' These views recall those of Whitaker (1933*b*) on the 'regulation' of respiration by fertilization. The idea that metabolic changes at fertilization may be positive or negative according to the past history of the unfertilized egg may seem startling, but there is evidence to support this idea. The fact that the increase in O_2 uptake after fertilization of *Psammechinus* eggs depends on the delay between spawning or removal from the ovary (Fig. 11) is one example, as is the paradoxical behaviour of *Urechis* eggs after fertilization (Tyler & Humason, 1937). Another concerns the contradictory results of Infantellina & La Grutta (1948) and

Bolognari (1952) on the glutathione content of sea-urchin eggs both before and immediately after fertilization. There are two morals to be drawn from these new 'difficulties' in interpreting metabolic changes at fertilization. First, the spectre of unreproducible or contradictory results, which often haunts the biologist, may not be so frightening as it sometimes seems; secondly, in investigating the various changes which occur at fertilization, we must not only bear in mind species differences, but also the past history of the material, for it is evident that the pre-fertilization history of eggs influences metabolism during early development.

Tricarboxylic acid cycle. The tricarboxylic acid cycle undoubtedly functions in sea-urchin, oyster (Cleland, 1950*b*), and certain fish eggs. Oxaloacetate, succinate, α-ketoglutarate, glutamate and citrate are rapidly oxidized (Crane & Keltch, 1949), while Keltch *et al.* (1950) showed that a particulate cell-free system obtained from unfertilized *Arbacia* eggs esterified orthophosphate during the oxidation of Krebs cycle intermediates. These results have been confirmed and extended by Yčas (1954), who also demonstrated the inhibition of O_2 uptake by malonate, and by Cleland & Rothschild (1952*b*), who showed that egg homogenates oxidize pyruvate, the effect on O_2 uptake being more pronounced when the formation of pyruvate from endogenous substrate was blocked by fluoride. The complete oxidation of pyruvate is cogent evidence in favour of the tricarboxylic acid cycle, as the latter is believed to be the only mechanism which will combust pyruvate to CO_2 and water in animal tissues. The oxidation of pyruvate by sea-urchin eggs was also noted by Goldinger & Barron (1946) and Krahl *et al.* (1942).

Hishida & Nakano (1954) found that addition of the usual Krebs cycle intermediates stimulated the O_2 uptake of egg homogenates of *Oryzias latipes*; succinate had the greatest effect, increasing the endogenous O_2 uptake by 500%. The O_2 uptake of these homogenates, with or without the addition of substrates, increases twenty-four hours after fertilization. The authors interpret this as showing that there is a synthesis of Krebs cycle enzymes at this time, though they point out that this view conflicts with that of Spiegelman & Steinbach (1945), who found that the O_2 uptake of frog's egg homogenates decreased as development went on. The latter concluded that, at the beginning of development, the respiratory enzymes in the egg were not saturated by their appro-

priate substrates, although at a later stage in development they were. This does not seem to be true in the case of the eggs of *Oryzias latipes*, in which the Japanese workers have found a pronounced synthesis of cytochrome oxidase and phosphothiamine during development.

Fluoroacetate has only a small inhibiting effect, about 20% at 10^{-2}M, on sea-urchin egg homogenates. As it is a powerful inhibitor of acetate metabolism in some animal tissues, acetate metabolism may only play a small part in the overall oxidative metabolism of such systems; but the student should remember that fluoroacetate inhibits the Krebs cycle at the citric acid stage, owing to its conversion to fluorocitric acid. Fluoroacetate will, therefore, inhibit any oxidation which is mediated through the Krebs cycle. The acetate inhibition hypothesis is confirmed by Hultin's experiments (1953*b*) on the metabolic utilization of 1-^{14}C-acetate in sea-urchin embryos. Utilization is low in the early phases of development, though the position is not the same later, when visible differentiation starts. This suggests that except perhaps during the first few minutes after fertilization, fatty acid metabolism may be of secondary importance in the early phases of development.

CHAPTER 7

METABOLIC AND OTHER CHANGES AT FERTILIZATION

This chapter consists of a list, with occasional notes, of the principal changes (or lack of changes) which have been reported to occur at, or soon after, fertilization. Morphological, structural and certain other changes are dealt with in separate chapters. The references are not intended to be all-inclusive and little attention has been paid to priority of discovery. References with an asterisk after them are brief notes. f. stands for fertilized or fertilization; u. for unfertilized.

(1) Increase in permeability to sparingly ionized solutes, e.g., water, $k = 0{\cdot}1$ (u.), 0·2–0·4 (f.). *A. punctulata* (R. S. Lillie, 1916; Lucké *et al.*, 1931).

The permeability constant k is the amount of water (μ^3), entering the cell in unit time (1 min.), through unit area (μ^2), under unit difference of osmotic pressure (1 atm.). The change in k occurs 2–4 min. after fertilization and is complete in 10 min. (Lucké & McCutcheon, 1932).

Measurements of permeability changes after fertilization or parthenogenetic activation of sea-urchin eggs have recently been made by Ishikawa (1954), Fig. 14. When the eggs of *Hemicentrotus pulcherrimus* are activated by treatment for 12 min. with sea water containing butyric acid (50 ml. sea water + 3 ml. N/10 butyric acid), there is, according to this worker, *no* breakdown of cortical granules, no membrane formation and no increase in permeability to water. This casts some doubt on the importance of granule breakdown (see pp. 9–10), which Ishikawa thinks is responsible for the increase in permeability, in activation. Similarly, Kusa (1953) was able to activate salmon eggs, parthenogenetically, without the cortical alveoli breaking down. Sugiyama (1953) says that butyric acid treatment does cause granule breakdown and membrane formation in the eggs of *Hemicentrotus pulcherrimus*. The shape of the normal fertilization curve in Fig. 14 is similar to that obtained by Hobson (1932*a*), using the eggs of *Psammechinus miliaris*.

(1·1) Osmotically inactive fraction, or 'non-swellable volume', 7·3% in u. and 27·4% in f. eggs. *A. punctulata* (Shapiro, 1948).

In this paper, Shapiro states that there is a 2·7% increase in the volume of this egg at fertilization, see p. 20.

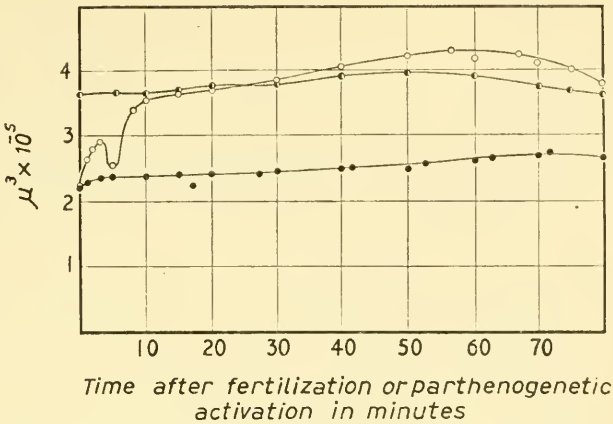


FIG. 14.—Change in volume of sea-urchin egg (*Hemicentrotus pulcherrimus*), in $\mu^3 \div 10^5$, after three minutes' immersion in 40% sea water + 60% distilled water, at various times after fertilization. The point whose co-ordinates on the middle curve are app. $2{\cdot}5 \times 10^{-5}$ μ^3 and 5 min. therefore refers to an egg which was placed in hypotonic sea water (for 3 min.), 5 min. after fertilization. The rate of swelling at this time is lower than it is in eggs which are examined earlier, or later, than 5 min. after fertilization. ○, normal fertilization; ◐, unfertilized eggs previously treated for 3 min. with M-urea; ●, unfertilized eggs previously treated with butyric acid-sea water for 30–50 secs. T° C., 17. After Ishikawa (1954).

(1·2) Negligible increase in permeability to water, $k = 0{\cdot}5$ (u.), 0·6 (f.). *C. variopedatus* (Shapiro, 1939*).

Compare the decline in O_2 uptake after fertilization in these eggs.

(1.3) 8% reduction in vapour pressure 30 min. after f. *R. temporaria* (Picken & Rothschild, 1948).

(2) Rate of P entry from external medium (to which ^{32}P had been added) 40 times greater in f. than in u. eggs. *A. punctulata* (Abelson, 1947*, 1948*).

The increase started 7–10 min. after f. $1{\cdot}6 \times 10^{-5}$M-4–6-dinitro-o-cresol inhibited P uptake by a factor of 6 though it doubled O_2 uptake and virtually inhibited division.

(2.1) Rate of P entry from external medium (to which ^{32}P had been added) 130–160 times as great in f. as in u. eggs (accumulation, not exchange). *S. purpuratus* (Brooks & Chambers, 1948*).

(2.2) No increase in rate of P entry from external medium (to

which ^{32}P had been added), after f., until after second cleavage, and then slowly. *U. caupo* (Brooks & Chambers, 1954).

These eggs are very variable in their respiratory response to fertilization (Tyler & Humason, 1937).

(2.3) Rate of P entry from external medium (to which ^{32}P had been added) and incorporation in acid labile fraction much faster after f. (accumulation, not exchange). *P. miliaris* (Lindberg, 1948).

(2.4) f. eggs accumulate P fast, 0·003–0·004 mg P/ml. f. eggs/min., from external medium (to which ^{32}P had been added). u. eggs do not, though P exchange occurs. Increase in ATP content 70–90 min. after f. at expense of inorganic P. *S. purpuratus*, *S. franciscanus*, *L. pictus* (Chambers & White, 1949*, 1954).

(2.5) No change in ATP content after f. (1·20–1·45 mg/ml. in *A. forbesi*). *S. droebachiensis*, *A. forbesi* (Chambers & Mende, 1953*a*).

Increased synthesis after fertilization might be balanced by increased utilization.

(2.6) ATPase activity in f. egg homogenates twice that in u. egg homogenates. *S. purpuratus* (Connors & Scheer, 1947).

This may be due to Ca release at fertilization (see 4).

(2.7) Increase in arginine phosphate and decrease, 32%, in inorganic phosphate, app. 5 min. after f., the latter being mainly accounted for by the former. No change in ATP. *S. droebachiensis* (Chambers & Mende, 1953*b*).

This paper contains a discussion of the contrary results obtained by Runnström (1933), Zielinski (1939), and Örström & Lindberg (1940), using the eggs of *Paracentrotus lividus*. The authors conclude that the change in inorganic P might have taken place when these eggs were fertilized, but that, because of their higher inorganic P content (× 15–30), it might have been missed. The synthesis of arginine phosphate occurs precisely at the time of the transient increase in O_2 uptake and acid production, see Fig. 13. Although the authors suggest that the energy for the synthesis is derived from carbohydrates, the low R.Q. at this time is difficult to reconcile with this view. The authors also made one interesting observation on an abnormally concentrated egg suspension, in which the eggs developed pathologically. In this case, there was no decrease in inorganic P and no synthesis of arginine phosphate after fertilization.

(2.8) Intracellular distribution of ^{32}P before and after f. *L. pictus* (Whiteley, 1949).

When the egg is centrifuged (E. B. Harvey, 1941), it separates into two fragments: a large light part containing an oil cap, a small hyaline layer, the nucleus or mitotic figure and yolk; and a smaller heavier part, containing mitochondria and an optically empty layer. In unfertilized eggs which have been allowed to accumulate ^{32}P for six hours, there is 1–2 times as much ^{32}P in the light, as in an equal volume of the heavy part. In fertilized eggs, the ratio (^{32}P light)/(^{32}P heavy) = 0·5, implying that a good deal of the P goes into the mitochondria, about 20 minutes after fertilization. This paper contains a review of the work on P metabolism in eggs up to 1949.

(3) Readily exchangeable K, 20% of total in u. eggs; 80% in f. eggs. *S. purpuratus* (Chambers *et al.*, 1948*).

(3.1) K exchange 16 times faster in f. than in u. eggs. *S. purpuratus* (Chambers, 1949*).

(3.2) Cyclical changes in K permeability after f. Uptake, 0–10 min.; release, 10–40 min.; uptake, 40–60 min. 60% of K in eggs non-exchangeable. *P. lividus*, *A. lixula* (Monroy-Oddo & Esposito, 1951).

These results are statistically significant.

(4) Bound Ca decreases at f. *A. punctulata* (Heilbrunn *et al.*, 1934; Mazia, 1937).

(4.1) Ca diffuses out of eggs at f. *P. lividus* (Örström & Örström, 1942).

(4.2) Ca and Mg diffuse out of eggs at f. (first measurements 15 min. after f.). *A. lixula* (Monroy-Oddo, 1946).

Lindvall & Carsjö (1951) were unable to confirm (4.1) and (4.2) using the eggs of *Echinus esculentus*; neither were Barnes, Cleland and I (unpublished). The presence of small amounts of jelly, which contains Ca, round unfertilized eggs, may be a confusing factor in experiments of this type. The uptake of Ca by sea-urchin egg jelly has been described by Rudenberg (1953); (4.1) and (4.2) should not be accepted unless they are confirmed. There is a further discussion of the role of Ca in fertilization in chapter 8.

(5) The following changes in inorganic constituents occur at f.

(or laying?): Na, −10%; Ca, −8%; Cl, −8%; inorganic P, −16% (conversion to organic P?). *S. salar* (Hayes *et al.*, 1946).

Manery & Irving (1935) found no change in Cl when the eggs of *Salmo gairdneri* Richardson were fertilized.

(6) Increase in rate of disappearance of pyruvate, added to sea water, after f.; u., 64 γ, f., 448 γ/hour/g. dry weight. *A. punctulata* (Goldinger & Barron, 1946).

This experiment does not, in fact, tell one anything about the differences between u. and f. eggs in regard to pyruvate metabolism. The authors do not refer to the changes in permeability which are known to occur when sea-urchin eggs are fertilized. Nor did they examine the possibility of different effects on f. and u. eggs of lithium, which was added to the sea water as lithium pyruvate. This paper contains several interesting but speculative observations about fertilization, some of which are now known to be wrong. The earlier negative results on pyruvate metabolism of Runnström (1933) and Örström & Lindberg (1940) are said to be due to inadequate techniques.

(7) No change in diphosphothiamine at f. *A. punctulata* (Krahl *et al.*, 1942; Goldinger & Barron, 1946).

The first authors found 8·5 γ/g. dry weight and the second 16·2 γ/g. dry weight. Diphosphothiamine is concerned in pyruvate metabolism, as it acts as a coenzyme in the reaction:

$$CH_3{\cdot}CO{\cdot}COOH \xrightarrow[\text{diphosphothiamine}]{\text{carboxylase}} CH_3CHO + CO_2$$

(7·1) Fall in free thiamine and phosphothiamine synthesis after f. *O. latipes* (Hishida & Nakano, 1954).

(8) DPN, u., 385 γ/g. wet eggs; f. (30 min.), 345; f. (600 min.), 242. *S. franciscanus*, *A. punctulata* (Jandorf & Krahl, 1942).

(9) Decrease in ribonuclease activity after f. *A. punctulata* (Bernstein, 1949*).

(9·1) DNA of f. eggs not formed from RNA of u. eggs. *A. punctulata* (Schmidt *et al.*, 1948; Villee *et al.*, 1949).

(9.2) DNA of f. eggs formed from RNA of u. eggs. *P. lividus* (Brachet, 1933).

It is now generally agreed (Abrams, 1951) that (9.2) is wrong and (9.1) is right. RNA fragments may, however, be utilized in DNA synthesis.

(9.3) Surface of oil droplets Feulgen-negative in u. eggs and Feulgen-positive in f. eggs. *N. succinea* (Lovelace, 1949*).

This experiment requires confirmation. A Feulgen-positive reaction in the presence of lipids is a common artifact. However, Costello (1938) found that the oil droplets can be made to coalesce after fertilization, but not before, in eggs of the same species.

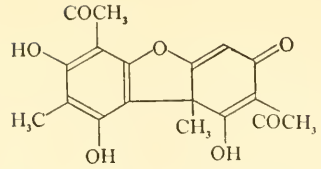
(9.4) Doubling of DNA content of pronuclei after f. and before fusion. *M. differentialis*, *P. variegatus*, *M. musculus* (Swift, 1953; Swift, unpubl.).

(9.5) Appearance of RNA 'granules' in the cytoplasm of the spermatozoon and their dispersion in the egg cytoplasm by the end of maturation. *A. equorum* (Panijel, 1947; Pasteels, 1948).

(9.6) 'Dissolution' of Ascaridin, previously surrounding the sperm head. *A. equorum* (Pasteels, 1948).

Note on (9.1)–(9.6). The problem of RNA and DNA variations and their inter-relationship in eggs and spermatozoa is extremely complicated, if not confused; the non-specialist might be wise to wait a few years before tackling this question. Pasteels & Lison (1951) for example, made a detailed examination of the DNA content of the eggs and spermatozoa of *Sabellaria alveolata*. Contrary to expectation, they claimed first, that the DNA content of eggs and spermatozoa was not the same; and secondly, that the DNA content of diploid cells was not double that of haploid cells, the ratio being 1 : 12 in the case of spermatozoa compared with the first blastomere, and 1 : 5·5 in the case of the mature egg compared with the first blastomere. After fertilization, Pasteels & Lison found that DNA was synthesized in the male pronucleus, its content increasing by a factor of 6. The factor for the egg during this period was 2·75. Further information will be found in a paper by Alfert & Swift (1953), who could not confirm the results of Lison & Pasteels; in a review by Swift (1953); and in a paper by Hoff-Jorgensen (1954), who reported that the *total* DNA content of sea-urchin eggs (*Paracentrotus lividus*) and of frogs' eggs (*Rana temporaria* Linn.) remained constant for a considerable time (3 and 18 hours, respectively) after fertilization.

(9·7) D-usnic acid inhibits the fusion of the male and female pronuclei, though they approach each other in the usual way. It also inhibits cleavage and the uptake of ^{32}P, though O_2 uptake is unaffected. *A. punctulata* (Marshak & Fager, 1950).

Usnic acid,  is believed to inhibit

DN-ase when cobalt is present, though it is also thought to interfere with oxidative phosphorylation. As several workers have put forward the view that the biological activity of unsaturated carbonyl compounds is due to their additive interaction with thiol compounds (Sexton, 1949), the effects of cysteine and thioglycollic acid on usnic acid inhibition would be of interest. The fusion of the pronuclei is also inhibited by anaerobiosis and HCN (Runnström, 1935*a*).

(10) Increase in alkaline phosphatase after f. *P. lividus* (Wicklund, 1948).

(10.1) No change in alkaline phosphatase until 10 hours after f. *P. miliaris* (Gustafson & Hasselberg, 1950).

(10·2) No change in acid or alkaline phosphatase until gastrulation. *A. punctulata* (Mazia *et al.*, 1948*).

(10.3) No sudden change in alkaline phosphatase after f. *X. laevis*, *S. mexicanum* (Krugelis, 1950).

(11) Transient NH_3 production at f. *P. lividus* (Örström, 1941).

(11.1) Non-transient NH_3 production after f. *A. punctulata* (Hutchens *et al.*, 1942).

This work has been criticised on technical grounds by Lindberg (1945).

(11.2) f. eggs form glutamine from added NH_3 and glutamic acid. u. eggs do not. *P. lividus* (Örström, 1941).

(12) No change in 'free' hypoxanthine and guanine, present in the ratio 2: 1. *R. temporaria* (Steinert, 1952).

(13) No change in free 'cholesterol' at f. *E. cordatum* (Ohman, 1945).

(13.1) No change in free 'cholesterol' for 15 min. after f.; then gradual decrease. *P. lividus* (Lindvall & Carsjö, 1948).

(13.2) Increase in free 'cholesterol' at f. *A. lixula* (Monroy & Ruffo, 1945*).

(13.3) Fall in free cephalin at f. *E. cordatum* (Ohman, 1945).

(13.4) Decrease in lipids after f. *A. punctulata* (Hayes, 1938).

(13.5) No change in lipids or lipo-proteins after f. *A. punctulata* (Parpart, 1941*).

(13.6) Increase in lipids after f. *A. equorum* (Panijel, 1951).

(14) Decrease in carbohydrate after f. *P. lividus* (Zielinski, 1939); *P. lividus*, *E. cordatum* (Örström & Lindberg, 1940); sea-urchin (Lindberg, 1945); *A. equorum* (Panijel 1951); *R. temporaria* (Panijel, 1951); *P. miliaris* (Lindberg, 1943).

(14.1) No decrease in carbohydrate after f. *A. punctulata* (Hutchens *et al.*, 1942).

This work has been criticised by Lindberg (1945).

(15) No change in dipeptidase activity after f. *P. miliaris*, (Doyle, 1938); *E. parma* (Holter, 1936).

(15.1) Slight decrease in dipeptidase activity after f. *U. caupo* (Linderstrøm-Lang & Holter, 1933).

(15.2) Fall in amino-nitrogen, principally glycine, during first two hours after f. and accumulation of free glutamine immediately after f. *P. lividus* (Gustafson & Hjelte, 1951).

(15·3) No change in free amino acids after f. *S. purpuratus* (Berg, 1950*b*).

(15·4) Amino acid metabolism and, in particular, Kavanau's recent results, are discussed in chapter 6.

(15.5) Transient activation of proteolytic enzymes. *P. lividus*, *A. lixula*, *E. cordatum*, *E. esculentus*, *B. lyrifera* (Lundblad, 1949–1954).

Lundblad has published an interesting series of papers showing that proteolytic enzymes are activated transiently at fertilization (Fig. 15). Two of these enzymes, EI and EIII, can be activated in unfertilized egg homogenates by ribonuclease. Lundblad & Hultin (1954) believe that EI and EIII are activated at fertilization by ribonuclease, derived from the fertilizing spermatozoon or from the egg, following the activation of egg ribonuclease by the fertilizing spermatozoon. EI and EIII are also activated by SH groups, which have an inhibitory effect on EII, as does heparin. These observations are not

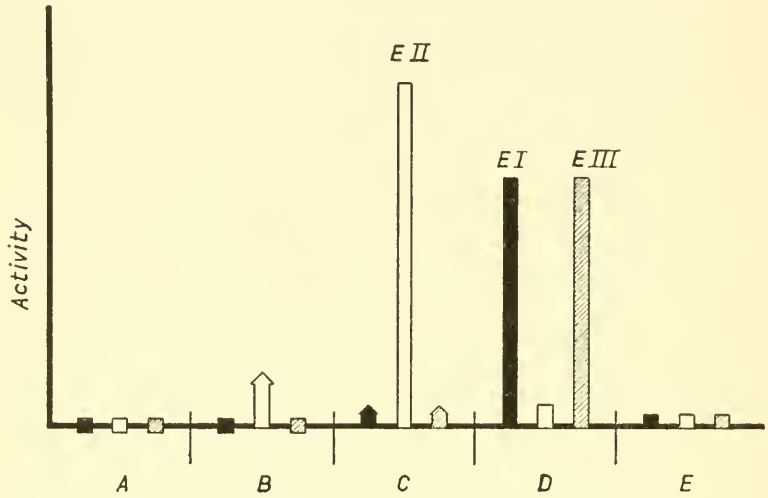


FIG. 15.—Activity of proteolytic enzymes in sea-urchin eggs. *A*, before fertilization; *B*, at fertilization; *C*, at membrane elevation; *D*, immediately after membrane elevation; *E*, ten minutes after membrane elevation. Three enzymes, *EI*, *EII* and *EIII*, are involved in each period. After Lundblad (1954*d*).

incompatible with the ribonuclease hypothesis, as SH reagents increase the activity of ribonuclease in liver (Roth, 1953), while heparin inactivates sea-urchin egg ribonuclease (Lindvall & Carsjö, 1954). The most dominating feature of Lundblad's experiments is the sharp activation of proteolytic enzymes during the elevation of the fertilization membrane. It would, therefore, be of interest to investigate the course of events in eggs treated with trypsin before fertilization, so that membrane formation is suppressed.

(16) Solubility of proteins in M-KCl decreases by 12%, 4–10 min. after f. *A. punctulata*, *S. purpuratus* (Mirsky, 1936).

(16.1) New electrophoretic component appears in water extracts shortly after f. and disappears after 30 min. Ammonium

sulphate extracts show a decrease in solubility of one component immediately after f. *P. lividus* (Monroy, 1950).

In a later paper by Monroy & Monroy-Oddo (1951, p. 246), it is said that 'the electrophoretic patterns of the water extracts of unfertilized and fertilized eggs of *Arbacia lixula* are essentially identical. . .'

(16.2) No change in solubility of proteins extractable with M-KCl after f. *A. punctulata* (Monroy & Monroy-Oddo, 1951).

The eggs were treated with trypsin before fertilization to prevent the formation of the fertilization membrane, it being thought that the salts in the perivitelline fluid might cause ambiguous results. Although the authors found a change in the M-KCl-soluble fraction after fertilization of *Arbacia lixula* eggs, they say that further experiments are needed to decide whether the postulated coagulation 'is an actual occurrence under natural conditions.' (p. 253). Similar doubts about Mirsky's results have been expressed by Lindvall & Carsjö (1951), using the eggs of *Echinus esculentus*.

(17) Production of acetyl choline or acetyl choline-like substance at f. *P. depressus* (Numanoi, 1953*b*).

In the same paper the author states that spermatozoa of *Pseudocentrotus depressus* (A. Agassiz) and *Clypeaster japonicus* contain cholinesterase, as do mammalian spermatozoa (Sekine, 1951). Scheer (1945) showed that acetyl choline and physostigmine, at concentrations of $1/10^4$, inhibited the activation of eggs of *Urechis caupo* and *Strongylocentrotus purpuratus*. In a later paper, Scheer & Scheer (1947) found that the inhibitory action of acetyl choline was extremely complicated, at any rate in *Urechis* eggs, inhibition being most marked when activation was affected by subjecting unfertilized eggs to potassium- and magnesium-free, but calcium-enriched, sea water. In view of these results, it would be unwise to attach too much importance to Numanoi's results until they have been confirmed. Heilbrunn (1952, pp. 635–642) makes some cogent remarks about the necessity for caution in interpreting experiments designed to demonstrate the existence of acetyl choline in tissues.

(18) Release of sulphate due to splitting of heparin-like polysaccharide sulphate at egg surface (jelly?) by sperm sulphatase. *H. pulcherrimus* (Numanoi, 1953*a*).

(19) No change in hexokinase activity at f. *A. punctulata* (Krahl *et al.*, 1954*b*).

(20) No change in urease activity at f. *S. purpuratus* (Brookbank & Whiteley, 1954).

(21) Negligible change in catalatic activity after f. *E. esculentus* (Rothschild, 1950).

(22) Increase in glutathione content immediately after f; fall after 45 min. *P. lividus* (Infantellina & La Grutta, 1948).

(22.1) Fall in glutathione content immediately after f.; rise after 45 min. *P. lividus* (Bolognari, 1952).

(23) No change in refractive index of cytoplasm, 1·375, nor of egg nucleus, 1·360, after f. *P. miliaris* (Mitchison & Swann, 1953).

(24) O_2 uptake, see chapter 5.

(25) Acid production, see chapter 5.

CHAPTER 8

STRUCTURAL CHANGES AT FERTILIZATION

Birefringence. Chapter 1, The Morphology of Fertilization, contains a description of various structures which can be seen in fertilized and unfertilized eggs, with an ordinary microscope. Studies with the polarising microscope provide information from which a few inferences can be made about the sub-microscopic morphology of eggs, the scale changing from microns to ångstroms. The most interesting of these studies concerns the egg cortex. In the unfertilized sea-urchin egg, this structure is negatively birefringent with respect to the tangent and has a radial optical axis (Runnström *et al.*, 1944*b*). The birefringence is very weak but as the cortex also scatters and depolarises a considerable amount of light, the egg surface appears quite bright under the polarising microscope. At fertilization this negative birefringence disappears, at about the same time as the light-scattering properties of the egg surface change (chapters 1 & 9). Some minutes later, the cortical birefringence re-appears, reaching a maximum by anaphase. According to Mitchison & Swann (1952), the intrinsic birefringence is more strongly negative than the total birefringence, so that the form birefringence must be positive. This suggests that the molecular structure of the cortex is radial, but that the micellar organisation is tangential. The fall in total birefringence at fertilization is probably due to a decrease in radial molecular order.

Both the fertilization membrane and the hyaline layer are birefringent. The former is virtually isotropic when first formed, but by the time it is fully tanned (pp. 9–10), its form and intrinsic birefringence are positive (Runnström, 1928; Runnström *et al.*, 1946). The birefringence of the hyaline layer is positive with respect to the tangent (Runnström *et al.*, 1944*b*).

Mechanical properties of the cortex. Many of the methods of investigating changes in the physical state of the cortex and the cytoplasm suffer from the disadvantage that they do not enable an estimate to be made of the degree to which measurements of cortical viscosity, surface tension, or rigidity are influenced by the

physical state of the cytoplasm, and *vice versa*. Cole, for example, attempted in 1932 to measure the internal pressure of a sea-urchin egg, and its surface tension, by observing the changes in the shape of the egg when compressed by a small gold beam. Reliable measurements by this method require rather large deformations of the egg. One then measures the resistance to deformation of internal structures, such as the amphiaster, as well as the resistance to deformation of the cortex. Mitchison & Swann (1954*a*, *b*; 1955) invented an instrument, which they call the Cell Elastimeter, though it is commonly known as the 'Sucker', to overcome such difficulties. The instrument consists of a micropipette, filled with water, one end of which is connected to a device for producing

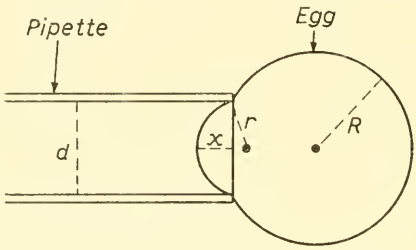


FIG. 16.—Diagram showing measurements involved in estimating cortical stiffness with the Elastimeter. After Mitchison & Swann (1954*a*).

negative pressures; the other end, which has a terminal diameter of about 50 μ, is placed against the surface of an egg. When a negative hydrostatic pressure is applied, part of the egg surface bulges into the pipette, Fig. 16. From a knowledge of the diameter of the pipette, the negative pressure applied, and the degree to which the egg bulges into the pipette, an estimate can be made of the tension at the surface or, more accurately, the stiffness, of the cortex. The measurements involved in estimating the degree of bulging are shown in Fig. 16. The units for stiffness are dynes.cm^{-2} per μ deformation. By comparing the observed behaviour of sea-urchin eggs and large-scale models such as balloons, Mitchison & Swann came to two conclusions, apart from quantitative data referred to later; first, as was to be expected, that the cortex was elastic; and secondly, that its thickness was appreciable. Mitchison (1956) has adduced other, optical, evidence in favour of this view. The permeability barrier may still, of course, be fairly thin, the capacitance measurements mentioned in chapter 10

suggesting a layer of the order of 100 Å thick for this part of the cortex. If magnified, a sea-urchin egg would therefore be more like a tennis ball, which resists deformation because of the rigidity of its 'cell membrane', than like a rubber balloon, which resists deformation because of its internal pressure. Measurements are expressed in terms of corrected stiffness, which involves correcting observations for variations in egg and pipette diameters. Full details are given in Mitchison & Swann (1954*a*). Experiments with the Elastimeter show that the Young's modulus of the cortex of the unfertilized sea-urchin egg is $1–2.10^4$ dynes.cm^{-2}, and that the internal pressure of the egg is less than 95 dynes.cm^{-2} (Mitchison & Swann, 1954*b*).* These values agree rather well with those of E. N. Harvey (1931), who measured the centrifugal force necessary to split a sea-urchin egg into two halves.

What happens, structurally, to the cortex at and after fertilization? Mitchison & Swann (1955) found that, at fertilization, there was a sudden rise in stiffness, from the value 9 dynes.cm^{-2}.μ^{-1} for the unfertilized egg, which was followed by a fall, during the early sperm aster stage, to the lowest value, 4 dynes.cm^{-2}.μ^{-1}, which occurs during development. There is a steep rise, to about 61 dynes.cm^{-2}.μ^{-1}, in late anaphase, just before cleavage. Normal eggs cannot be used for direct measurements of stiffness *at* fertilization, because of the elevation of the fertilization membrane. Runnström *et al.* (1944*b*) discovered, however, that treatment of unfertilized sea-urchin eggs with trypsin prevented the elevation of the fertilization membrane, perhaps by digesting the vitelline membrane. Treatment of unfertilized eggs of *Psammechinus microtuberculatus* (de Blainville) with 0·1% w/v. trypsin in sea water reduced the stiffness of the cortex of these eggs, which normally is 9 dynes. cm^{-2}.μ^{-1}, by a factor of 4 or 5. At fertilization, the cortical stiffness increased by the same factor, Fig. 17.

In their most recent paper, Mitchison & Swann (1955) give a comprehensive review of the earlier methods of investigating the mechanical properties of the cortex. More or less serious objections can be raised against all the classical methods. Cole's experiments have already been discussed; in the same way, measurements of the ease with which eggs can be separated into two halves by

* Young's modulus can be derived from the corrected stiffness values, assuming a cortical thickness of 1·6 μ and zero internal pressure. The internal pressure is very low.

centrifugation are open to the criticism that the measurements are sensitive to changes in cytoplasmic gelation as well as to the structural changes in the cortex, which are the object of the enquiry. In spite of difficulties in estimating the internal pressure of eggs, upon which interpretation of Elastimeter measurements depends to a considerable extent, this technique provides a powerful and

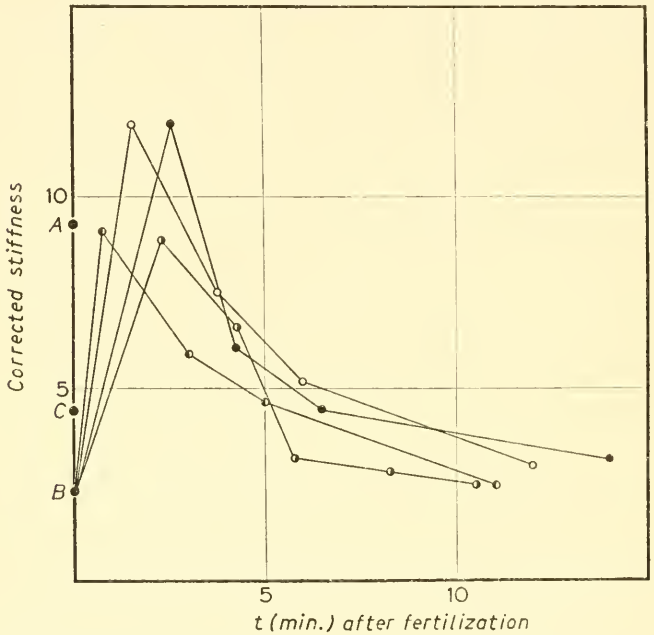


FIG. 17.—Changes in cortical stiffness (corrected, in dynes.cm^{-2} per μ deformation), at fertilization. The four curves refer to four eggs of *Psammechinus microtuberculatus*, previously treated with trypsin to prevent elevation of the fertilization membrane. *A*, value for stiffness of normal unfertilized egg; *B*, value for stiffness of trypsin-treated unfertilized egg; *C*, stiffness of fertilized egg at sperm aster stage. After Mitchison & Swann (1955).

sensitive new method of investigating the structural characteristics of the cortex.

Calcium and protoplasmic consistency. Heilbrunn (1915, 1952) has for many years been the principal proponent of the view that cellular reactivity in general and activation in particular are 'caused' by the release into the cell interior of calcium, previously bound in the cortex in Ca-protein complexes. Once released, calcium promotes a protoplasmic clotting reaction or gelation. The release of

calcium from the egg cortex is associated with the so-called liquefaction or decrease in rigidity of this structure, which follows fertilization or parthenogenetic activation. Heilbrunn believes that this rigidity is due to the presence of calcium in the cortex; but according to Mitchison & Swann (1955), removal of calcium from the external medium makes very little difference to the rigidity of the cortex of the fertilized sea-urchin egg (bearing in mind that this treatment removes the hyaline layer), while in the case of the unfertilized egg, calcium lack causes a slight, but insignificant, increase in rigidity. W. L. Wilson (1951) examined the post-fertilization changes in the cortical properties of the eggs of *Chaetopterus variopedatus*. He expressed rigidity in terms of the centrifugal force, which, applied for 1 minute, was necessary to break the continuous layer of granules which exists in the cortex of this egg, in not less than 16 and not more than 19 eggs, out of a sample of 20. The experiment was done at different times after fertilization. Wilson found that the rigidity of the cortex decreased after fertilization, for about 4 minutes. After 6 minutes, the rigidity began to increase and in 10–11 minutes, it had reached the pre-fertilization level, where it stayed until 35 minutes after fertilization, when it again declined. These observations do not fit well with the results of the more refined measurements of Mitchison and Swann, though this may be explained by the different maturation states of the eggs of *Chaetopterus* and of the sea-urchin at fertilization. Alternatively, Wilson may have been measuring the Shear modulus, and not Young's modulus, which Mitchison and Swann measured. Until 30 minutes after fertilization, Wilson's results are to a certain extent reflected in the changes in cytoplasmic viscosity * which occur in the same egg during the same time. Heilbrunn & Wilson's results (1948) on this latter subject, which are, perhaps, less open to doubt than those of Wilson on cortical changes, are reproduced in Fig. 18. Similar curves were obtained for the eggs of *Arbacia punctulata* and *Cumingia tellinoides* by Heilbrunn in 1921. It is not quite clear how the observed reduction in cytoplasmic viscosity, immediately after fertilization, fits in with Heilbrunn's thesis that one of the important characteristics of egg activation is cytoplasmic clotting or gelation, due to the release of calcium from the cortex. According to Heilbrunn, a wide variety

* The cytoplasmic viscosity of an unfertilized egg is about 3 times that of water (Heilbrunn, 1952).

of agents, such as heat, cold and electric shocks, which cause parthenogenetic activation, induce a release of calcium from the cortex, which in turn starts a cytoplasmic clotting reaction. In confirmation of this view, Shaver (1949) found that the foreign agents which must be present as 'needle contaminants' for successful traumatic parthenogenesis in frogs' eggs, favour blood-clotting, while the introduction of the anticoagulant heparin * into the egg inhibits parthenogenesis. Harding (1949) claimed to have confirmed Shaver's results and stated, in addition (1951), that Shear's bacterial

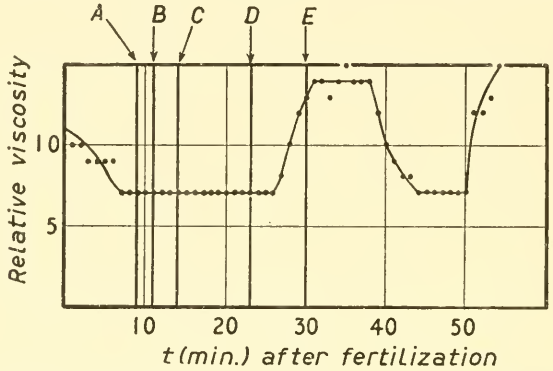


FIG. 18.—Changes in the viscosity of the egg of *Chaetopterus variopedatus*, at various times after fertilization. The viscosity units are arbitrary, being the number of seconds of centrifugation at 2325 g. required to achieve a particular accumulation of fat granules at the centripetal pole of the egg. *A*, first polar body; *B*, late anaphase; *C*, 2nd maturation prophase; *D*, 2nd polar body; *E*, apposition of pronuclei. After Heilbrunn & Wilson (1948).

polysaccharide, which inhibits blood-clotting, also inhibited parthenogenesis in the frog's egg. Calcium is a co-factor in that part of the blood-clotting reaction which is concerned with the production of thrombin and Heilbrunn believes that the release of calcium into the egg cytoplasm promotes the production of a thrombin-like substance. Although there is a little doubt that fertilization and parthenogenetic activation induce changes in the distribution of calcium in the egg and that some form of cortical liquefaction (see also the reference on p. 6 to Allen's work), followed by cytoplasmic gelation, occurs when an egg is activated, the comparative significance of these reactions is not yet clear. Are they, as Heilbrunn believes, *the* important feature in fertiliza-

* Heparin is not only an anticoagulant, but is, for example, also concerned with the dissolution of chylomicrons in blood plasma (Florey, 1955).

tion, in the way that the action potential might be said to be the important feature in nervous reactivity; or are calcium release, cortical liquefaction and cytoplasmic gelation just some of the many manifestations of the differences between fertilized and unfertilized eggs, which will only be seen in correct perspective many years hence? In any case, is it legitimate or logical to talk about *the* important feature in any biological reaction? Is the action potential more important than the liberation of acetyl choline at a nerve junction? Such questions are meaningless. The most we can do, and all we need to do, in describing some biological phenomenon, is (ultimately) to make a time-sequential analysis of the phenomenon in the language of physical chemistry. If calcium release comes first in the fertilization reaction, we can, if we wish, define coming first in terms of prime importance.

The idea that gelation is one of the characteristics of the early phases of fertilization is supported by the work of Immers (1949) on the inhibitory action of fertilizin on the coagulation of fibrinogen by thrombin. This, together with the observation that periodate counteracts the anticoagulating action of fertilizin and heparin and, in some circumstances, facilitates fertilization (Runnström & Kriszat, 1950), has from time to time led to the expression of the view that fertilizin inhibits fertilization and that the spermatozoon introduces into the egg, or releases in the egg, a factor which overcomes the inhibition imposed by fertilizin. This sort of argument does not bear much examination. In so far as an unfertilized egg is not a fertilized one and that morphological observations for more than a hundred years have shown that fertilization unleashes all sorts of anabolic reactions which are obviously catalyzed by enzymes, the mere statement that the unfertilized egg is in an inhibited condition and that enzyme systems are activated at fertilization is only a re-statement of the morphological facts. What we must do is to identify the activated enzyme systems and the chemical nature of the so-called inhibition. As regards the former, a little progress has been made; in the case of the latter, the idea that fertilizin is responsible is a little difficult to swallow.

Apart from being concerned in cortical liquefaction and cytoplasmic gelation, calcium is important in other ways in fertilization and in the physiology of the gametes. In marine invertebrates, it must be present in the external medium for fertilization or, for that matter, the fertilizin–antifertilizin reaction, to take place at

all, as Loeb pointed out in 1914. Calcium is also involved in the maturation of marine eggs; this has been noted in several different sorts of immature eggs, as Table 15 shows.

TABLE 15

Presence of Ca in the external medium necessary for the induction of maturation

Organism	*Phylum*	*Reference*
M. glacialis	Echinodermata	Dalcq, 1924
T. neptuni	Annelida	Hobson, 1928
P. strombi	Annelida	Dalcq *et al.*, 1936
H. uncinatus	Annelida	Pasteels, 1935
P. triqueter	Annelida	Hörstadius, 1923
N. succinea	Annelida	Heilbrunn & Wilbur, 1937
B. candida	Mollusca	Pasteels, 1938
S. solidissima	Mollusca	Heilbrunn & Wilbur, 1937

Calcium is necessary for the discharge of the cortical granules in the sea-urchin egg (Moser, 1939*b*); for the transformation of the isotropic cortical granules into birefringent rods while in the perivitelline space (Endo, 1952); and for the tanning of the fertilization membrane (Hobson, 1932*b*), though for reasons discussed in chapter 1, this may be another and less detailed way of expressing Endo's observation. In addition, Hultin (1949, 1950*a*, *b*) has made an interesting series of studies on the effects of adding calcium to sea-urchin egg homogenates prepared in Ca-free media. His main findings were that, after adding $CaCl_2$, there was:

(1) A rapid uptake of O_2, without a corresponding increase in CO_2 evolution, for twenty minutes. Cyanide did not inhibit the reaction.

(2) No breakdown of carbohydrate.

(3) Inhibition of the reaction after treatment of homogenates for thirty minutes with 0·005 M-monoiodoacetate.

(4) Granular components in the homogenates, including yolk globules, disintegrated explosively and there was a marked increase in the viscosity of the whole system. *Note.* Echinochrome granules, which are freely distributed in the cytoplasm of the unfertilized egg of *Arbacia punctulata*, move into the cortex after fertilization, arriving there about ten minutes after fertilization (E. N. Harvey, 1910). Heilbrunn (1934) showed that these pigment granules disintegrate explosively in the presence of free calcium or magnesium, the former being a hundred times as

efficacious as the latter. When equal quantities of sea water and of isotonic potassium chloride, oxalate, or citrate are added to fertilized eggs of *Arbacia punctulata*, eleven, but not less than eleven, minutes after fertilization (i.e. at the monaster stage), the echinochrome granules explode and echinochrome is found in the surrounding sea water (Churney & Moser, 1940). The eggs subsequently cleave in the usual way. Heilbrunn says that this experiment demonstrates the release by potassium of calcium, previously bound in the cortex. These observations may have some bearing on the 'clotting reaction', as Donnellon (1938) observed that, during the clotting of sea-urchin perivisceral fluid, the red granules in the red amoebocytes and the colourless ones in the white amoebocytes explode. Isotonic KCl has the same effect (cf. exploding platelets in mammalian blood-clotting.)

(5) Acid production, which was not inhibited by iodoacetate to the same extent as O_2 uptake. The addition of papain, instead of $CaCl_2$, also induced acid formation.

These striking observations indicate once more the importance of calcium in the metabolism of eggs. Moreover, they confirm, as Heilbrunn has so often insisted, that changes in the distribution of calcium have profound effects on protoplasmic structure.

The effects of changes in the external environment on the cortex. An interesting contribution to our knowledge of the cyclical changes in cortical structure which occur after fertilization and parthenogenetic activation was made by Herlant in 1920, using the eggs of *Paracentrotus* (*lividus*?) and *Sphaerechinus* (*granularis* (Lamarck)?). Herlant examined two things: the incidence of plasmolysis (shrinking) and cytolysis in eggs exposed to various hypertonic solutions; and the variation in the susceptibility of eggs to a number of cytolytic agents, at various times after fertilization. As Herlant's paper is extremely long and does not contain a summary, its contents are summarised below:

(1) Fertilized sea-urchin eggs undergo cyclical variations in their susceptibility to hypertonic sea water (100 ml. s.w. + 20–25 ml. 2·5M-NaCl), as indicated by plasmolysis. Unfertilized eggs plasmolyse, the surface of the egg becoming wrinkled, but this characteristic progressively disappears after fertilization, until there is a zero plasmolysis from 5–25 minutes after fertilization. From 25–70 minutes, plasmolysis re-occurs, becoming maximal

after 45 minutes and remaining so until 75 minutes after fertilization, when mitosis begins (17° C.). During mitosis there is no plasmolysis. This description of the reactions of fertilized sea-urchin eggs to hypertonic sea water is somewhat surprising and does not agree with the descriptions of Hobson (1932*a*) and Monroy & Montalenti (1947); the experiments, which are technically simple, could be repeated with advantage. Interpretation, however, is not easy, because of the delay between treatment and the onset of symptoms.

(2) In grossly hypertonic sea water (60 ml. s.w. + 40 ml. 2·5M–NaCl), the eggs either plasmolyse or cytolyse, the incidence of the former being inversely proportional to that of the latter.

(3) If it is assumed, as Herlant does, that changes in the external environment directly and exclusively affect the properties of the cortex, NaCl- and KCl-enriched sea water increase the permeability of the cortex, while $CaCl_2$ and $MgCl_2$ have the reverse effect. According to Herlant, an increase in the incidence of plasmolysis implies a decrease in permeability to salts, it being assumed that permeability to water is unaffected by the experimental treatment; conversely, that an increase in cytolysis is caused by an increase in permeability to ions. Tracer work will clarify these questions in the next few years.

(4) Herlant also investigated the effects of various agents on the incidence of cytolysis in fertilized and unfertilized sea-urchin egg suspensions; his results are summarised in Fig. 19.

Herlant's experiments show without doubt that fertilization induces a series of cyclical changes in the properties of the cortex.* For example, the low resistance of the egg to digitonin in the interval 10–35 minutes after fertilization suggests that, just before this period, cholesterol or a cholesterol-like compound becomes more accessible to the digitonin molecule, at the cell surface. These and other results which may bear on the same subject are summarised in Table 16. When examining this table, it must be remembered that, with the possible exception of those of Mitchison & Swann (1954*a*), the methods for investigating changes in the

* Herlant was not, of course, the first person to make this discovery, as a perusal of R. S. Lillie's papers or Needham's *Chemical Embryology* shows. But his work was far more comprehensive than anything done before, or, probably since, (1955).

TABLE 16

Effects of fertilization on the reactions of sea-urchin eggs to various treatments

Treatment	2–10 *min. after f.*	10–35 *min. after f.*	40–55 *min. after f.*	*References*
Weak hypertonic s.w.[1] (+ NaCl)	Spherical plasmolysis [2]	Polyhedral plasmolysis [3]	Spherical plasmolysis	Runnström, 1924; Runnström *et al.*, 1944*b*; Monroy & Montalenti, 1947
Strong hypertonic s.w. (+ NaCl)	Cytolysis	Plasmolysis	Cytolysis	Herlant, 1920
Hypotonic s.w.	High resistance	Low resistance	High resistance	Just, 1928; Page, 1929; Hobson, 1932*a*; Herlant, 1920
Saponin Digitonin Acetone	High resistance	Low resistance	High resistance	Herlant, 1920
Cortical rigidity (centrifugation) [4]	Falls	Increases	Falls	W. L. Wilson, 1951
Cortical rigidity (centrifugation) [4]	Increases	Falls	Increases	Marsland, 1939
Cortical stiffness (Elastimeter) [4]	Increases, then falls	Slowly increases	Sharp increase	Mitchison & Swann, 1955
Heat (45° C)	No blisters	Blisters	Few blisters	Ohman, 1945
Birefringence of cortex [5]	None	Re-appears [6]	Low	Monroy, 1945; Monroy & Montalenti, 1947; Mitchison & Swann, 1952

Notes

1, s.w., sea water; 2,  ; 3,  ; considerable variation is found in different species (see, for example, Hobson, 1932*a*). 4, discussed in detail in this chapter; 5, Swann (1952) pointed out that the tangentially negative (= positive radial) birefringence of the cortex, which is pronounced in the unfertilized egg (Runnström *et al.*, 1944*b*), is sometimes confused with the birefringence of the hyaline layer, which is of the opposite sign and may obscure the cortical birefringence. 6, the findings of various workers on the correlation between the type of plasmolysis and cortical birefringence are not entirely consistent. Some of the discrepancies may be due to variations in the behaviour of different batches of eggs, or to species differences; but there still remain some ambiguous points which require clarification.

consistency of the cortex may not be unequivocal, in the sense of being able to separate cortical from cytoplasmic changes. Table 16 is of interest in several different fields of cell physiology. First, it presents biological evidence of structural and, probably, metabolic changes in the cortex, induced by activation; secondly, it provides a number of clues for the further investigation of the cortex; and

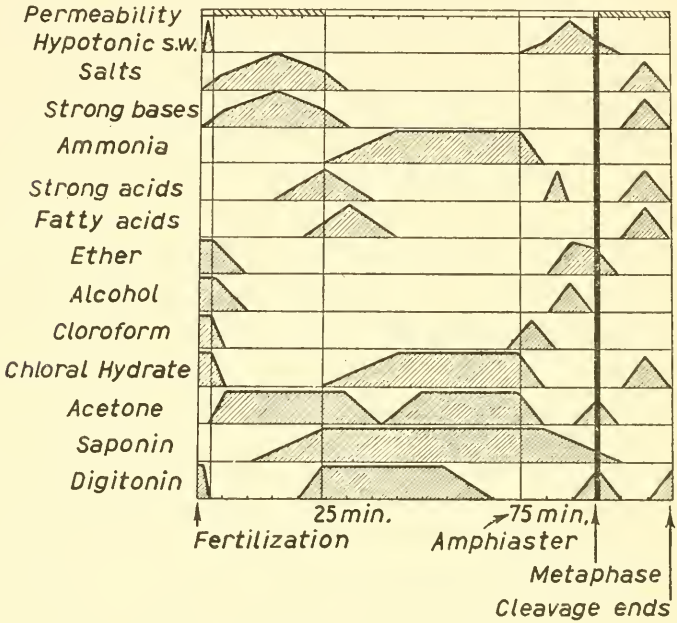


FIG. 19.—Changes in the susceptibility of the sea-urchin egg to various reagents. Each horizontal section shows the percentage cytolysis at different times after fertilization. The hatched regions at the top are periods of high permeability. After Herlant (1920).

thirdly, the information may not only apply to the fertilized egg, but to other dividing cells. Now that we understand a little more about cell membranes and, with the aid of labelled atoms, can investigate sodium, potassium, phosphorus and, perhaps, calcium fluxes across the cell surface, more rapid progress should be possible in this field, in spite of the complications introduced by the possibility of active transport. Among other things, it would be interesting to examine the effects, if any, of saponin and digitonin on the form and intrinsic birefringence of the cortex, in the ways described by Mitchison & Swann (1952).

CHAPTER 9

POLYSPERMY

After fertilization the haploid egg nucleus normally fuses with a single haploid sperm nucleus: in the rare event of it uniting with more than one sperm nucleus, development is almost invariably abnormal. Two mechanisms exist to prevent polyandrous syngamy of egg and sperm nuclei. One of these, Type I Inhibition, prevents all but one spermatozoon from entering the egg. In the second mechanism, Type II Inhibition, several spermatozoa enter the egg but only one sperm nucleus unites with the egg nucleus.

Type II Inhibition of Polyspermy. The eggs of selachians (Rückert, 1899), urodeles (Jordan, 1893), reptiles (Oppel, 1892), Polyzoa (Bryozoa) (Bonnevie, 1907), birds (Blount, 1909; Hamilton, 1952), some molluscs (Bretschneider, 1948), and many insects (Richards & Miller, 1937) are normally polyspermic in the sense that several spermatozoa enter the egg at fertilization. Only one of these fuses with the female nucleus, the remainder degenerating near the surface of the egg, though abortive divisions of the supernumerary sperm nuclei sometimes occur. Fankhauser (1925–1948) has made a most interesting study of polyspermy in the eggs of *Triturus helveticus* (Razoumowsky) and of *Diemictylus viridescens* (Rafinesque), in which several spermatozoa usually enter the egg after insemination. In spite of polyspermy, cleavage is normal and monospermic, provided less than ten spermatozoa enter the egg, although for three hours after fertilization the egg appears to be typically and pathologically polyspermic. At the time when *the* sperm nucleus (the one which happens to be nearest the egg nucleus after maturation) fuses with the egg nucleus, the supernumerary sperm nuclei, which may have proceeded as far as prophase or even to the release of chromosomes (particularly in *Diemictylus viridescens*), begin to degenerate, those nearest the fusion nucleus degenerating first. If, after fertilization, an egg is ligatured so that one half contains the egg nucleus and sperm nuclei, the half in question develops normally. The other half, which contains sperm nuclei but no egg nucleus, cleaves as frequently as a

normal egg, but the cleavages are usually abnormal and retarded. Nuclear and astral cycles are often out of phase in dividing supernumerary sperm complexes. In the half of a previously fertilized egg which contains the egg nucleus but no sperm nucleus, cleavage is incomplete and anastral. When a half contains no egg nucleus, cleavage is frequently abortive. If one half has neither egg nor sperm nucleus, there is either no cleavage or abortive cleavage. When a fertilized egg is constricted, *not* ligatured, so that it becomes shaped like a dumb-bell, the situation becomes more complicated, but can be summarized as follows, calling one half of the dumb-bell L and the other R.

L, female nucleus and sperm; R, sperm; thin bridge between L and R. Cleavage in L and R.

L, female nucleus and sperm; R, sperm; thick bridge between L and R. Cleavage in L but not in R.

L, female nucleus; R, sperm. No cleavage in L, cleavage in R. When the bridge is thick, the male or female nucleus sometimes moves across the bridge and joins the other nucleus, in which case a normal first cleavage furrow occurs in L or R.

These experiments suggest that substances may diffuse out of the female nucleus or *the* sperm nucleus under the influence of proximity to the female nucleus, which cause degeneration of supernumerary spermatozoa. Some information about the characteristics of this substance might be obtained by continuing Fankhauser's studies with different bridge widths between dumb-bells and by observing the variations in the times at which supernumerary sperm nuclei degenerate according to their distance from the fusion nucleus. These remarks apply particularly to the eggs of *Triturus helveticus.* In those of *Diemictylus viridescens*, supernumerary sperm nuclei which are at different distances from the fusion nucleus begin to degenerate at the same time, a phenomenon which seems to be incompatible with a diffusion mechanism. The experiments of Allen (1954), on the behaviour of the egg nucleus after fertilization of sea-urchin eggs sucked into narrow glass capillaries, suggest that some substance diffuses out of the sperm head or male pronucleus into the cytoplasm, where it affects the female pronucleus. Allen found that the latter elongated or bulged in the direction of the sperm head, which at the time was located at the periphery of the elongated egg. To make distinctions

between physical and chemical mechanisms in biological systems, except in the case of such phenomena as electrotonus, is of questionable value; but for what this distinction is worth, Ziegler (1898), who also did constriction experiments with cotton threads on sea-urchin eggs, found that if he constricted the fertilized egg in such a way that the spermatozoon was in one part, connected by a narrow bridge of cytoplasm to the rest of the egg, which contained the female nucleus, that part of the egg which contained the female nucleus failed to divide though the nucleus showed signs of activity, while the part containing the sperm head did divide. A somewhat similar situation occurs in the eggs of *Crepidula plana* Say, in which only that part of the egg which contains the sperm nucleus divides, the other part being called, rather tautologically, a polar body. Evidently one of the essential features in fertilization is the introduction of a division centre into the egg by the spermatozoon, 'rather than a diffuse chemical action of the sperm' (T. H. Morgan, 1927, p. 512). In recent years little work has been done on the problems raised by Type II Inhibition of polyspermic development. Further experiments on newt eggs, which are easily obtained, would bring their own reward.

Failure of the Inhibition. Type I and Type II Inhibition may fail or be induced to fail, and an immense amount of work was done on the consequences of such failures in the nineteenth and early twentieth centuries (see, for example, Boveri, 1907). When more than one sperm nucleus engages in syngamy, abnormal cleavages occur, followed by the early death of the pathological embryo. Some cases of dispermic adults are, however, known in insects (Goldschmidt & Katsuki, 1931; L. V. Morgan, 1929), and in birds (Hollander, 1949), in which large patches of feathers, coloured differently from the rest of the animal, are found on occasions. Interesting accounts of two 'mosaic' cocks, as such animals are called, bred from the same sex-linked cross, Light Sussex ♀ × Rhode Island ♂, and of a mosaic daughter of a mosaic cock, will be found in papers by Greenwood & Blyth (1951) and Blyth (1954). These mosaics may sometimes be caused by dispermic fertilization, in which case part of the tissue of the animal has a set of genes derived from one spermatozoon and part from a different set of genes derived from the other spermatozoon. The evidence for the existence of dispermic adult humans, or indeed any mammals, is not conclusive.

Polyspermy in Mammals. Except in the case of *Ornithorhynchus anatinus* (Shaw & Nodder), in which it may be that polyspermy is the rule rather than the exception (Gatenby & Hill, 1924), the incidence of polyspermy in mammalian eggs is low, being of the order of 1–2% under normal mating conditions. They should therefore be classified as Type I eggs, in which only one spermatozoon enters. Austin & Braden (1953) published an important paper, which includes a critical review of earlier work, on polyspermy in rats and rabbits. They found that rat and rabbit eggs go through a critical period, depending on the time after ovulation when they come into contact with spermatozoa, as regards their ability to prevent polyspermy. If mating is delayed so that the eggs remain unusually long in the female reproductive tract before fertilization, the incidence of polyspermy goes up quite sharply, which recalls the increases in polyspermy observed in ageing marine invertebrate eggs. These observations, together with the small numbers of polyspermic eggs normally found, confirm that mammalian eggs belong to the Type I class. Although, for obvious reasons, Austin & Braden were unable to obtain any information about the ultimate fate of polyspermic mammalian eggs, they observed that the supernumerary male pronuclei did not induce the formation of separate spindles near the periphery of the egg, but approached the female pronucleus and contributed to the first cleavage spindle. The spindle, however, was normal and not multipolar. These observations raise a number of questions about the fate of accessory chromosomes, but the answers must await the results of further experiments. A valuable summary of the information available about the occurrence of polyspermy in mammalian eggs has recently been published by Braden *et al.* (1954).

Type I Inhibition of Polyspermy. Type I Inhibition, often called the Block to Polyspermy, involves a change in the egg surface, after the fertilizing spermatozoon has become attached, such that further spermatozoa cannot enter the egg. It has been known since the nineteenth century that the fertilizing spermatozoon initiates some alteration in the egg which prevents re-fertilization, and many workers have observed changes in the morphology of the egg surface immediately after fertilization, such that a point on the surface opposite to where the fertilizing spermatozoon became attached is the last to be affected. The wave of granule breakdown

in the eggs of *Arbacia punctulata* at fertilization, observed by Moser (1939*a*), and the similar phenomenon observed by Endo (1952) in Japanese sea-urchins, are examples of such morphological changes. Some workers, for example Runnström (1928, p. 395), have reported that when the egg is examined with dark ground illumination, a colour change passes over the egg surface in four to six seconds. But they did not realize that unless the egg is fertilized at the equator, as seen by the observer who is looking down the microscope, estimates of the time taken for the cortical change to pass completely over the egg have no meaning. The observer, whose eye is directly above the north pole of the egg, sees it in optical section, the periphery of the section being the circumference of an equatorial great circle. When a spermatozoon fertilizes an egg, a cortical change spreads out in all directions over the egg surface, from the point of attachment of the spermatozoon

FIG. 20.—Development of cortical change in an egg fertilized at 1.30 o'clock (Rothschild & Swann, 1949).

(Fig. 20). If the spermatozoon fertilizes the egg exactly at the north pole, the whole periphery of the optical section of the egg will alter instantaneously, because the cortical change reaches all points on the equatorial great circle simultaneously. This is shown in Fig. 21, where the periphery of the alteration in surface structure is shown as a series of 'isochrones' depicting the position of the leading edge of the change at various times after fertilization has started. If the spermatozoon fertilizes the egg at the equator, the periphery of the optical section changes colour at the true rate of propagation. At points intermediate between the north (or south) pole and the equator, the propagation rate, as judged by the time taken for the periphery of the optical section to change colour, will seem to be faster than it actually is. The form of the conduction–time curve will also be affected by these considerations. The best way to make a serious examination of the conduction time of the surface change is by taking dark-ground cinemicrographs of eggs, immediately after insemination, and noting in which eggs a fertilization cone can be seen at the equator. When

the cone is seen at the equator, the egg was fertilized at the equator. An experiment of this sort shows that the conduction time is about twenty seconds at 18° C in the eggs of *Psammechinus miliaris* (Rothschild & Swann, 1949), with quite a significant induction period before the cortical change begins to pass over the egg surface (Kacser, 1955). It is natural to ask whether the change in surface structure underlying this colour change or increase in light scattering is the block to polyspermy, but this at once raises another question—how many spermatozoa collide with the egg and, in particular, with

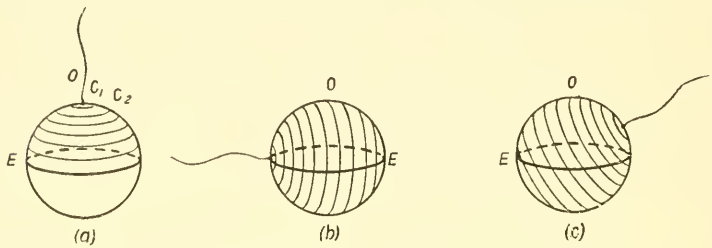


FIG. 21.—Passage of the cortical change over the egg surface. The observer at *O* sees the egg in optical section, the periphery of the section being the equatorial great circle *E*. (*a*) Fertilization at the north pole. The whole of the cortical great circle *E* changes colour instantaneously. (*b*) Fertilization at the equator. The cortical great circle *E* changes colour at the true rate at which the change is propagated. (*c*) Fertilization at 2.0 o'clock. The change affects the cortical great circle *E* more quickly than the whole egg and the conduction rate appears to the observer to be higher than it is. The concentric circles c_1, c_2, etc. represent the leading edge of the propagated change at times t_1, t_2, etc. (Rothschild & Swann, 1949).

parts of the egg surface unaffected by the surface change until the end of the twenty second period? When eggs are inseminated with fairly dense sperm suspensions, 10^7/ml., swarms of spermatozoa are normally seen round every egg in the suspension; yet only one spermatozoon fertilizes each egg. This has led people to believe that the block to polyspermy passes over the egg in an incredibly short time. But this presupposes that every spermatozoon which collides with an egg is capable of fertilizing it, and this presupposition requires examination.

The number Z of spermatozoa, moving in random directions—sea-urchin spermatozoa do move in random directions, even when near homologous eggs (Rothschild & Swann, 1949)—which will collide with an egg each second is given approximately by the equation

$$Z = \pi a^2 n \bar{c} \quad . \quad . \quad . \quad . \quad (1)$$

where a = radius of the egg, n = no. of sperm/ml., and $\bar{c}$ = mean speed of the spermatozoa. When the appropriate values are substituted in this equation, Z, the number of sperm-egg collisions per second, is found to be 0·16, 1·6, and 16 for sperm densities of 10^5, 10^6, and 10^7 per ml., respectively. These values for Z are probably too high because no account is taken of dead spermatozoa in the suspensions. Although the percentage of dead sperm in mammalian suspensions can be estimated by the live–dead staining technique (Lasley *et al.*, 1942), and is often about 10%, no method is at present available for doing this in suspensions of sea-urchin spermatozoa. As the cortical change takes twenty seconds to pass over the egg surface, there will in that time be respectively 3·2, 32 and 320 collisions at the three sperm densities in question. About half of these will collide with parts of the egg surface already covered by the cortical change, so that at a sperm density of 10^7/ml., only 1/160th of the spermatozoa colliding with the egg surface are capable of fertilizing it, *if* the cortical change is the block to polyspermy. How can the probability, p, of a sperm-egg collision being successful, be estimated? Elementary probability theory shows,* and experiments confirm, that if unfertilized eggs and spermatozoa are left in contact with each other for a series of known sperm-egg interaction times t_1, t_2, . . . t_n secs. ($n \not> 45$), and the number of fertilized and unfertilized eggs are later counted in each case, the proportion of unfertilized eggs, u, is given by the equation

$$\log u = -\alpha t \quad . \quad . \quad . \quad (2)$$

where α is the sperm-egg interaction rate or fertilization parameter with dimensions $[T]^{-1}$.

Assuming that the spermatozoa do not change their fertilizing capacity during the experimental period, α is a measure of the receptivity of the egg surface to spermatozoa and its value may reflect the fact that, on a submicroscopic scale, an egg surface is

* Divide the interval (o, t) during which the eggs and spermatozoa are in contact into r sub-intervals, each of duration τ. Let the probability of an egg being fertilized during τ be p, and of not being fertilized, q, where $p + q = 1$. There being r sub-intervals of duration τ during (o, t), the probability of an egg not being fertilized throughout (o, t) is, by the Product Theorem

$$q^r = \exp(r \log q) = \exp(t/\tau . \log q)$$
$$= \exp(-\alpha t)$$
where $\alpha = -1/\tau . \log q$

The theoretical proportions of unfertilized and fertilized eggs are, therefore, $u = \exp(-\alpha t)$ and $f = 1 - \exp(-\alpha t)$.

probably a mosaic of sperm-receptive and non-receptive regions. It can be expressed, approximately, in the form $\alpha = Zp$, where p is the probability of a successful sperm-egg collision. Some biologists have felt uncomfortable about treating spermatozoa as gas molecules colliding elastically with spheres. In defence of this feeling, α, though more abstract, has more 'depth' than p and Z. For if we only make the plausible and weak assumption that the chance of an egg being fertilized in a time interval δt is proportional to that interval of time, the proportionality constant being denoted by α, Equ. (2) automatically follows. Another advantage of α over p and Z is that it does not involve such considerations as the random movements of spermatozoa, chemotaxis, and the trap action of egg jelly on spermatozoa. Excluding chemotaxis, these factors interfere, though perhaps not to a great extent, with the 'sperm-gas molecule' analogy.

Calculations based on Equ. (2) show that at sperm densities of $7{\cdot}4 \times 10^4$/ml., $p = 0{\cdot}23$ and at a density of $9{\cdot}6 \times 10^6$/ml., $p = 0{\cdot}01$. Further enquiries into the conduction time of the block to polyspermy can be made by means of the following pair of experiments, run at the same time (Rothschild & Swann, 1951). In Exp. 1 unfertilized eggs were mixed with spermatozoa at $t = 0$ and functionally separated after 25 seconds, by killing the spermatozoa but not the eggs. The same procedure was adopted in Exp. 2, but, at the time when the spermatozoa were killed in Exp. 1, more spermatozoa were added, the sperm density being increased by a factor of 100. From the experiment described immediately above Equ. (2), it is known what proportion of eggs will have been fertilized in 25 seconds (Exp. 1), at any given sperm density, and therefore what proportion of the eggs will have started their blocks to polyspermy during that time. If, therefore, instead of killing the spermatozoa at $t = 25$, the number of sperm-egg collisions is greatly increased by the addition of more spermatozoa, there should be a negligible incidence of polyspermy unless a considerable number of successful sperm-egg collisions do occur during the block to polyspermy. Table 17 shows that, in such an experiment, there are three times as many polyspermic eggs in Exp. 2 as there were unfertilized eggs in Exp. 1. In other words, nearly half of the eggs which were fertilized in 25 seconds had not finished propagating their blocks to polyspermy in that time, and became polyspermic because of the new lot of collisions they received after the initial

25 second period was terminated. This experiment provides strong evidence for the conduction time of the block to polyspermy being of the order of seconds rather than small fractions of a second, as used to be thought. At the same time there remains the question as to why, if the block to polyspermy takes seconds to pass over the egg surface, so few eggs are normally polyspermic even at comparatively high sperm densities. This difficulty can be resolved by experiments based on the following self-evident proposition. Suppose, for example only, that a number of eggs are all fertilized at $t = 0$ and that the block to polyspermy is complete at $t = 5$ seconds.

TABLE 17

Conduction velocity of block to polyspermy (Rothschild & Swann, 1951)

Time, sec.	*Exp.* 1	*Exp.* 2
0 25	Sperm added, 3×10^6/ml. Sperm killed	Sperm added, 3×10^6/ml. More sperm added, 3×10^8/ml.
Unfertilized, % Monospermic, % Polyspermic, %	13 85 2	2 54 44

After 5 seconds there will be a a certain number of polyspermic eggs in the egg population. The eggs will not all be polyspermic because some of them will not have sustained more than one successful sperm-egg collision during that 5 seconds. As, however, all the blocks to polyspermy are complete by $t = 5$, the number of polyspermic eggs will never be greater than it is at $t = 5$. If the percentage of polyspermic eggs is 50 after 5 seconds, the percentage will still be 50 after 6, 60, or 600 seconds. It follows from this argument that, if we take a series of egg suspensions and fertilize all the eggs in them at $t = 0$, and then 'remove' the spermatozoa (Rothschild & Swann, 1951; Hagström & Hagström, 1954*a*) from these suspensions at various times after $t = 0$, for example, at 5, 10, 15 and 40 seconds, the time after which there is no increase in the incidence of polyspermy will be the conduction time of the block to polyspermy. Conversely, any decline in the incidence of polyspermy at $t = r$, as compared with $t = s$, where $r > s$, will be due to sampling errors or mistakes in deciding

whether an egg is polyspermic or monospermic. Fig. 22 (Rothschild & Swann, 1952) shows the sort of curve obtained when this experiment is done. In fourteen experiments of this type, the average figure for the conduction time of the block to polyspermy was 63 seconds. This type of experiment not only enables one to measure the time taken for the egg to become completely impermeable to spermatozoa after fertilization; it also enables estimates to

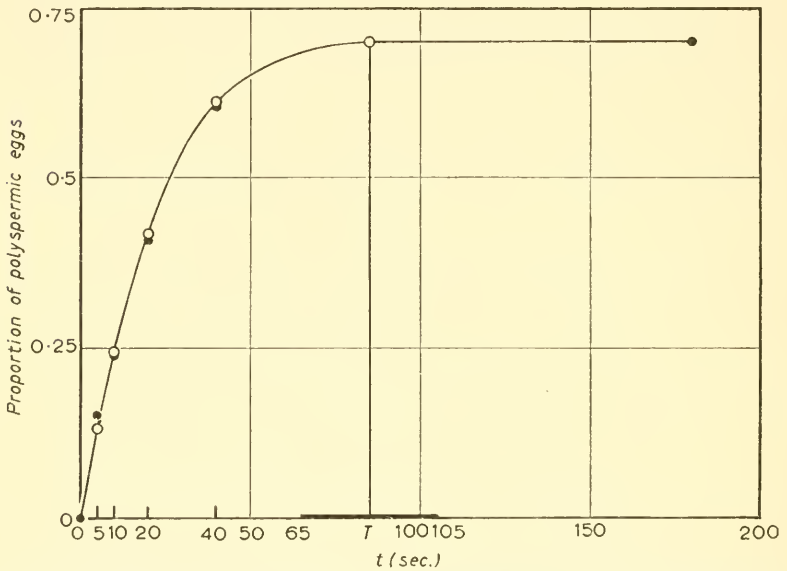


FIG. 22.—Proportion of polyspermic eggs (*Psammechinus miliaris*) in a suspension after various times of contact between eggs fertilized at $t = 0$, and spermatozoa. Sperm density, $9{\cdot}11 \times 10^7$/ml. ●, experimental points; ○, theoretical points. T, 85 sec. The thick line from 65–105 sec. on the time axis is the interval within which T lies with a fiducial probability of 0·9 (Rothschild & Swann, 1952).

be made of α during the passage of the 63-second change. During this time, α is only 1/20th of what it is before the first fertilization, which means that the receptivity of the egg surface is twenty times higher before than after the first fertilization. After the 63 seconds, the receptivity of the egg surface is zero. The implications of these observations are shown in Fig. 23, from which it will be seen that the block to polyspermy is probably diphasic, in the sense that a partial block to polyspermy sweeps over the egg surface in a second or so, and is followed by a slower mopping-up process which makes the egg completely impermeable to spermatozoa.

The cortical change which can be seen under the microscope is, therefore, not *the* block to polyspermy, but a reflection or phase of the slow part of it. The recent experiments of Nakano (1954) support this analysis.

Professor M. Sugiyama told me about an interesting experiment he had done, the details of which will be published. It supports the view that fertilization induces the propagation of an invisible change, distinct from the cortical change, round the egg surface or through the cytoplasm. He sucked an unfertilized sea-urchin egg into a glass capillary whose diameter was slightly less than that of

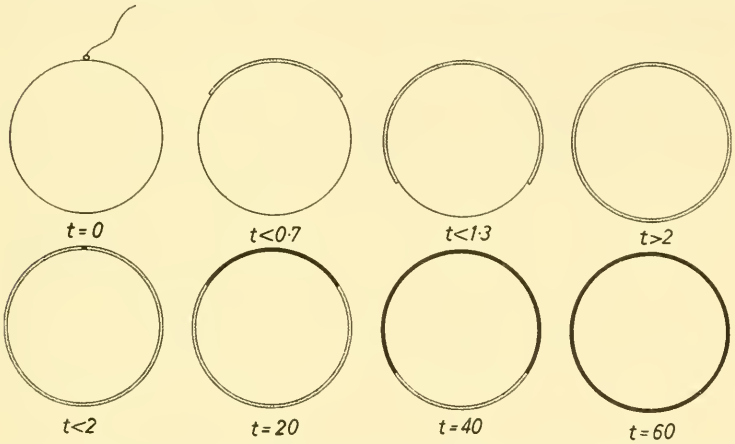


FIG. 23.—Diagram of the block to polyspermy in a sea-urchin egg, showing rapid partial block (grey), and slow complete block (black). Time, *t*, in seconds (Rothschild, 1953).

the egg. Part of the egg, the 'proximal' part, protruded from the end of the capillary, while the other, 'distal', part was within the capillary and in contact with sea water. When the capillary and egg were placed in M-urea in distilled water, there was a breakdown of granules in the cortex of the proximal part, but no membrane formation, because of the absence of divalent cations in the medium. A normal membrane formed round the distal part. Sugiyama considers that, in these conditions, both the invisible and cortical changes occur. When the capillary and egg were placed in sea water containing butyric acid, cortical granules broke down and a membrane formed at the distal end; but no breakdown of cortical granules occurred at the proximal end. Sugiyama's inter-

pretation is that butyric acid treatment by itself induces the invisible change, but inhibits the cortical change. When the capillary and egg were placed in sea water containing detergent, a membrane appeared round the proximal, but not the distal, part of the egg. The detergent induced the breakdown of cortical granules *without* the invisible change.

Type I Inhibition seems to involve more complicated mechanisms in mammalian eggs. When a rat spermatozoon passes through the *zona pellucida* of a rat egg, a minute slit or hole remains where the spermatozoon penetrated (Austin, 1951*b*). Braden *et al.* (1954) examined the relative positions of these slits in dispermic rat eggs, and obtained the distribution shown in Table 18, which shows that

TABLE 18

Angle, subtended at the centre of a dispermic rat egg, by two sperm slits in the zona pellucida (*Braden* et al., *1954*)

Angle, degrees	*No. of eggs*
20–30	2
40–50	2
80–90	4
100–110	10
120–130	7
140–150	3
160–170	1

the most probable place for a second spermatozoon to penetrate the *zona* is in the opposite hemisphere to that which the first spermatozoon penetrated. Braden and his co-workers conclude from this and other information about the number of spermatozoa entering eggs and the perivitelline space that the first spermatozoon to penetrate the *zona* initiates a self-propagating structural change, analogous to the cortical block to polyspermy, in the *zona*. By a probabilistic analysis which is somewhat similar to that used in the earlier calculations to do with the conduction time of the cortical block to polyspermy, they estimate that the conduction time of the change in the structure of the *zona*, which makes it impermeable to spermatozoa (see p. 12), is between 10 and 90 minutes. If this interpretation of the facts is correct, mammalian eggs have *two* blocks to polyspermy, one propagated round the cortex and the other round the *zona pellucida*. The latter appears to be a rather inefficient mechanism, if the minimum conduction

time is 10 minutes; but the implications of this inefficiency depend on the sperm density in the neighbourhood of the eggs and, therefore, on the sperm-egg collision frequency. If the collision frequency is low, a fast block to polyspermy is unnecessary and a simple calculation shows that if we assume an average sperm speed of 100 μ/sec. and the value 0·01 for the probability of a successful sperm-egg collision, a sperm density of the order of 4/μl, in the neighbourhood of the eggs, would not be excessive. But why should a mammalian egg need two distinct blocks to polyspermy?

An alternative interpretation of the experiments is worth consideration. In a number of non-mammalian eggs the cortical block to polyspermy is associated with the outward diffusion of substances into the perivitelline space, where they react with and tan the vitelline membrane (which is not, of course, always homologous with the *zona*). These substances are progressively released from the egg, following the progressive change in the egg cortex known as the block to polyspermy. It follows that the vitelline membrane is progressively tanned, though it is not excited, by the penetrating spermatozoon. The tanning is a passive process which results from the excitation, by a spermatozoon, of the egg cortex.

There are the following arguments against this hypothesis and in favour of the *zona* being capable of propagating its own tanning reaction in the rat egg:

(1) Cold-shock induces contraction of the egg and the release of fluid into the perivitelline space, both of which are characteristic of activation; but the *zona* remains permeable to spermatozoa (Austin & Braden, 1954*b*).

(2) Heat-shock inhibits the cortical block to polyspermy, but has little effect on the *zona* reaction (Austin & Braden, 1954*b*).

(3) After penetrating the *zona*, spermatozoa may remain for as long as thirty minutes in the perivitelline space before fertilizing the egg (Austin & Braden, 1954*a*). There should, therefore, be little relationship between the point of penetration through the *zona* and the point of attachment or fertilization on the egg surface.

The last argument seems the most cogent; but a more direct confirmation of the existence of this *zona* reaction would be welcome.

Induced Polyspermy. The possibility of interfering with the

block to polyspermy by chemical treatment of eggs was examined in detail by the Hertwigs in 1887. In recent years Clark (1936) is the only person who has made a systematic study of this subject. Although a number of different agents, which appear to have no common denominator, such as heat, cold, acid sea water, excess magnesium, alkaloids, fat solvents, chloral hydrate, and, in some circumstances, extracts from eggs and spermatozoa, cause polyspermy, nicotine and magnesium are probably the most efficacious

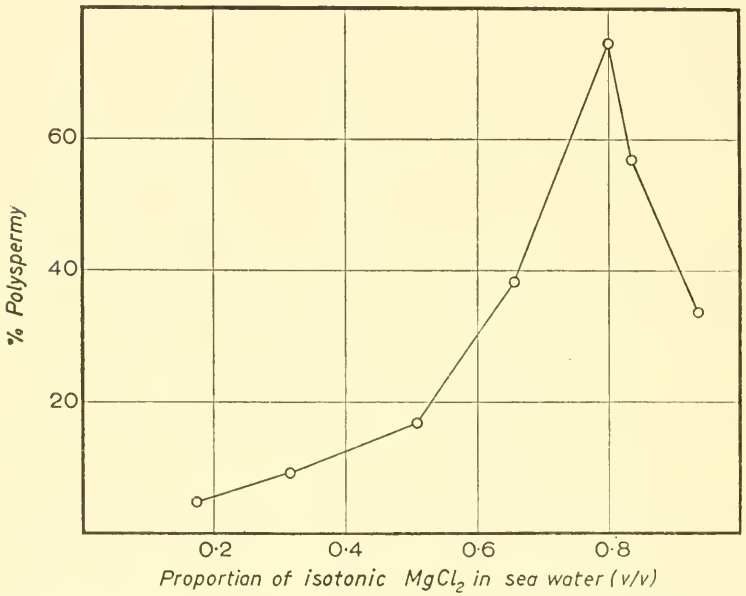


FIG. 24.—Effect of addition of isotonic $MgCl_2$ to sea water on incidence of polyspermy in eggs of *Arbacia punctulata* (after Clark, 1936).

agents. Fig. 24, showing the effect of increasing the magnesium content of sea water, is adapted from Clark's paper. As regards nicotine, Clark makes the interesting observation that the degree of polyspermy is a function of the time of exposure and the concentration of nicotine in the sea water. I have confirmed this observation, but it would be of interest (and comparatively easy) to examine the form of this strength-duration curve in greater detail. The existence of this relationship means that neither the block to polyspermy nor the receptivity of the egg surface is an all-or-none phenomenon; they are capable of being varied in a continuous way

over wide limits, quite apart from differences in threshold susceptibility between different egg batches. The question as to which of these two, α or the block, is influenced by nicotine can be examined by the method of known sperm-egg interaction times. The procedure is shown diagrammatically in Fig. 25. Such experiments show (Rothschild, 1953) that nicotine abolishes or slows up the fast partial block to polyspermy. According to the concentration of nicotine used and the duration of its application to the unfertilized egg, the conduction time of the nicotine-modified block to polyspermy can be extended to an almost indefinite extent.

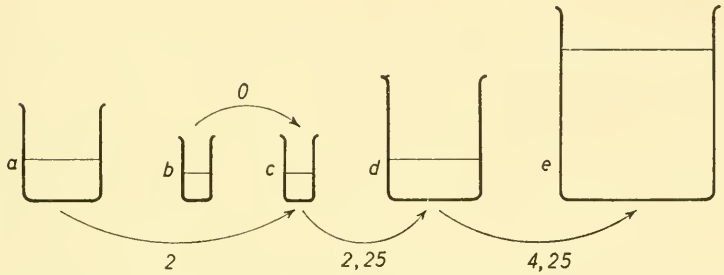


FIG. 25.—Experimental procedure for investigating effect of nicotine on the block to polyspermy. The curved lines with arrows show which vessels are emptied into which. The numbers by the curved lines refer to the times of emptying, e.g. 2, 25 means that vessel *c* was emptied into vessel *d* 2 min. 25 sec. after the beginning of the experiment, which started when *b* was emptied into *c* at $t = 0$. *a*, 10 ml. of sperm suspension; *b*, 2 ml. nicotine in sea water (1/1000, v/v); *c*, 2 ml. egg suspension; *d*, 90 ml. hypotonic sea water (28%); *e*, 700 ml. sea water + 21 ml. 11% NaCl in sea water (Rothschild, 1953).

Hagström & Allen (1956) have recently tried to re-examine the problem of nicotine-induced polyspermy in sea-urchin eggs, using the method of known sperm-egg interaction times. They conclude, on rather slender evidence, that the 20-second cortical change, described by Rothschild & Swann in 1949, is the block to polyspermy. Attractive as this idea may be, there is a considerable body of evidence which makes it unacceptable, quite apart from experiments with nicotine. Hagström & Allen have failed to appreciate the importance of this evidence. For example, in attempting to explain the fact that even when eggs are heavily inseminated, the proportion of polyspermic eggs is very low, they postulate that the acid produced by sea-urchin eggs at fertilization kills or inactivates supernumerary spermatozoa before they can

effect polyspermy. There is an obvious conceptual fallacy in this suggestion: the speed at which egg acid would have to be produced to achieve the desired result would be so high as to make egg acid production equivalent to a fast block to polyspermy. In the same paper these workers make the startling suggestion that the development of the hyaline layer plays a part in preventing polyspermy in normal eggs. They do not comment on one consequence of this suggestion: that if it is true, spermatozoa must be able to pass through the fertilization membrane. Until someone sees this occur, we need not consider whether a special mechanism (i.e. the hyaline layer) exists to obviate any ill effects from its occurrence.

Intracortical and intracytoplasmic conduction. There has been some discussion as to whether the cortical change (or the block to polyspermy) is conducted round the cortex or through the cytoplasm. After comparing the form of the curve showing the rate at which the sea-urchin egg surface becomes covered by the cortical change with two 'models', in which this was effected by intracortical and intracytoplasmic diffusion of a substance with molecular weight 20,000, I tentatively came to the conclusion (Rothschild, 1949*b*) that if a diffusion mechanism was involved, the evidence pointed towards the substance diffusing through the egg cytoplasm and hitting various points on the cortex from the inside, rather than towards diffusion in the cortex itself. Runnström & Kriszat (1952) came to the opposite conclusion on the basis of experiments done with damaged sea-urchin eggs. When these eggs become stuck to a glass surface and are subsequently fertilized, the part of the cortex which is stuck to the glass surface can be fertilized independently of the rest of the egg, when the egg is unstuck. They concluded that the cortex was injured by being stuck to the glass, that the cortical change was propagated round the cortex and that consequently the injured part remained unfertilized. The logical flaws in this argument are not difficult to see. If part of the cortex is upset by being stuck to a glass surface, it might be equally incapable of reacting to some stimulus from within as from neighbouring uninjured parts of the cortex. In any case, Hörstadius & Runnström (1953) have recently described experiments which might support the opposite viewpoint, intracytoplasmic conduction; as, if an egg is constricted in a glass tube so that it is nearly cylindrical in shape, and is fertilized at one end, a fertilization membrane appears at both free ends but not in

the middle. Although it has been known for some years that the cytoplasm of an egg alters in various ways immediately after fertilization, it seems probable that both mechanisms, a self-propagating cortical change as postulated by Allen (1954), and intracytoplasmic diffusion, occur. Whether one, both, or neither are uniquely responsible for the block to polyspermy is a question which still remains to be answered. Kacser (1955) has recently published an interesting and detailed examination of this question.

The experiments of Amoroso & Parkes (1947) lend support to the view that some substance in the sperm head may be concerned with the establishment of the block to polyspermy. They found a higher than normal incidence of polyspermy in the eggs of rabbits inseminated with x-irradiated spermatozoa (>2,500 r). The irradiation presumably inactivated some 'block-catalyst' in the sperm head, or modified some substance which normally diffuses out of the sperm head at fertilization and initiates the block to polyspermy. These experiments could be repeated with advantage, on a larger scale.

Reversal of Fertilization. I referred earlier to all-or-none reactions. Biologists have often hoped that the reactions of living matter would conform to this principle, but as time goes on, it is found that fewer and fewer do. Fertilization used to be thought of as an irreversible reaction. Once activated, the egg could not be reactivated. Tyler & Schultz (1932) were the first to cast doubts on this concept, when they found that fertilization could be inhibited and reversed in the eggs of *Urechis caupo* by treatment of the fertilized eggs with acid sea water. Reversal in this species, which is characterized by the egg reassuming its unfertilized appearance (Plate II) in spite of containing a spermatozoon, could only be achieved if the eggs were exposed to acid sea water within three minutes of fertilization. When such eggs are re-inseminated, a second spermatozoon penetrates and a normal block to polyspermy is established. More recently, Allen (1953), using the eggs of *Spisula solidissima*, reversed fertilization during the first four to five minutes after fertilization, by putting the eggs into calcium-free sea water, sea water acidified to pH 5, or sea water containing 0·3–0·5% ether. The most interesting experiments on this subject are those of Sugiyama (1951), using sea-urchin eggs, in particular those of *Hemicentrotus pulcherrimus*. Refertilization was achieved by subjecting fertilized eggs to calcium- and magnesium-free sea

water or molar urea, pH 7, with or without removal of fertilization membranes. Urea was found to be the more effective agent, and some of Sugiyama's results are given in Table 19. If eggs are re-

TABLE 19

Refertilization of sea-urchin eggs (Hemicentrotus pulcherrimus) *inseminated in normal sea water after washing in* M*-urea for 2 min. Membranes not removed before application of urea. Polyspermic eggs in control inseminations*, 0. T° C, 11.

Time from insemination to immersion in urea solution, sec.	*Monospermic eggs*	*Polyspermic eggs*
30	2	97
50	4	95
80	4	96
120	93	7

fertilized before anaphase, polyspermic divisions take place at the time of first cleavage; but if they are refertilized after the full growth of the amphiaster, first cleavage proceeds normally and polyspermy becomes evident at second cleavage. Eggs can even be refertilized after second cleavage. These experiments are of such interest that they deserve to be repeated, when some obscure points in Sugiyama's work could be cleared up. For example, no information is given in Table 19, which is extracted from Sugiyama's paper (p. 341), about refertilization sperm densities; but from other data in his paper, the final sperm dilutions must have been 1/100 or 1/1000. If the semen of this Japanese sea-urchin is similar to that of British varieties, these dilutions correspond to sperm densities of 2×10^8 and 2×10^7 per ml. The first of these is a fairly thick soup, and one wonders what would have happened if untreated fertilized eggs had been re-inseminated at this sperm density (cf. Table 17). What, if any, is the effect of supernatants from dense sperm suspensions on fertilized and treated eggs? Doubts may be entertained whether Sugiyama or Rothschild & Swann (1952), in their experiments involving insemination with dense sperm suspensions, have paid sufficient attention to Sampson's work (1926*a*, *b*) on the effects of sperm extracts on fertilization and development, though the point is discussed in Rothschild and Swann's paper (p. 479). This question certainly requires further investigation in the context of these recent experiments;

but interpretation will not be easy. The difficulties imposed by the use of high sperm densities did not arise in the experiments of Hagström & Hagström (1954*b*). They repeated Sugiyama's experiments but used sea-urchin eggs which had been pre-treated with trypsin, to remove the vitelline membrane, before fertilization. In these conditions refertilization can be achieved at a comparatively low sperm density, $2{\cdot}6 \times 10^6$/ml.

Conclusions regarding Type I Inhibition of Polyspermy. The experiments described in this chapter make it possible to construct a tentative picture, about which there will be disagreement, of the operation of the block to polyspermy (Type I). At the moment of attachment of the fertilizing spermatozoon to the egg surface, a change in cortical structure passes over the egg in less, but probably not much less, than two seconds. This reduces the chance of refertilization by a factor of twenty, and catalyses the production of a sperm-impermeable layer at the egg surface. Exploding cortical granules, discharging alveoli, or their equivalent near the egg surface may contribute to the formation of this layer, which is established in about sixty seconds, though this will undoubtedly vary from species to species. The integrity of the layer depends, *inter alia*, on the existence of divalent cations in the external medium. If they are not present, the layer may 'dissolve' and refertilization may be possible.

An action potential may pass over the egg surface before these changes, as so many cell physiologists have believed or hoped; but experiments to establish the existence of such action potentials, or electrical depolarization of the egg surface, are exceedingly difficult, and claims to have observed them must be examined with caution, if not scepticism. This subject is discussed in detail in the next chapter.

The block to polyspermy and the 'fertilization impulse', as the early phases of the fertilization reaction are sometimes called, are being actively investigated at the present time. Partial fertilization, for example, first systematically examined by Allen (1954), will undoubtedly shed light on these phenomena, and in a few years' time the picture referred to above will be less hazy, though it may require modification.

'*Heterologous*' *Polyspermy and Somatic Fertilization.* An account of polyspermy would be incomplete without some mention of recent Russian work on this subject and, in particular, of a very

interesting review by Kushner (1954). Kushner reports that if hens are inseminated on consecutive days by different and unrelated cocks, their offspring exhibit a higher growth rate, a higher live weight, a higher blood hæmoglobin content, and more vigour than control chicks obtained by 'normal' insemination. This is interpreted as being caused by 'heterologous' polyspermy (though only one male pronucleus fuses with the female pronucleus) and *not*, as might be thought, by heterosis. The supernumerary spermatozoa which enter the egg are said to influence its metabolism,* though it is also stated that the offspring resulting from mixed inseminations on occasions exhibit characteristics of both fathers. Similar experiments on pigs, sheep, cattle, trout, silkworms and silver foxes are reported, though in these cases, the accent is more on increased fertility and better post-natal performance after insemination with semen from different sires than on the appearance in the offspring of characteristics derived from both male parents. But it is almost as difficult to understand why, if one inseminates an animal with a mixture of semen from two sources, the offspring should be more vigorous than those obtained by insemination with either sort of semen singly, as it is to understand the phenomenon of tri- or poly-parental inheritance. Double inseminations would, of course, increase the number of spermatozoa in the female reproductive tract and this might increase the probability of fertilization. But this has nothing to do with the subsequent performance of the offspring unless polyspermy is a normal and, from the point of view of the future offspring, a valuable event. Similarly, it is possible, as Kushner says, that double matings increase the number of eggs ovulated, which might cause increased litter sizes in pigs. But again, in the absence of polyspermy, this has nothing to do with the later performance of the offspring.

Kushner also reports that the adverse effects of excessive inbreeding in rabbits can be counteracted by adding bull or ram semen to rabbit semen before its introduction into a doe.

It is hardly necessary to mention that these experiments, if confirmed, must have a profound effect on the whole subject of fertilization, quite apart from their impact on agriculture. The important thing is to repeat the experiments, which, in themselves, are

* Warburg (1911) and Brachet (1934*b*) have reported that the O_2 uptake of polyspermic eggs is slightly higher than that of monospermic eggs.

simple enough. Evaluation of the results, however, may not be so easy and it is interesting to find that Kushner, apart from interpreting the results of his colleagues on the basis of polyspermy, raises the question of somatic fertilization, a subject which, as he rightly points out, has fallen into disrepute in recent times, except in the special case of fertilization in sponges (Tuzet, 1950). Somatic fertilization implies that spermatozoa enter somatic cells in the female reproductive tract and exert an influence on them. Kushner quotes several Russian workers who have claimed that somatic fertilization of the mother exerts a beneficial effect on her offspring.

CHAPTER 10

BIOELECTRIC MEASUREMENTS

'*Action potentials.*' Cell physiologists have often hoped—and sometimes persuaded themselves to believe—that there is some common denominator in the responses of cells to stimuli. Heilbrunn (1952), for example, is a proponent of the 'calcium-release' theory of stimulation. According to this theory, such varied cells as muscle fibres, plant cells and unfertilized eggs respond to their specific stimuli by the intracellular release of calcium. R. S. Lillie (1924) placed the emphasis elsewhere. He believed that the first and most important response of the unfertilized egg to activation was the propagation of an action potential over the egg surface. Such an action potential would probably differ from those which occur in nerve fibres and cylindrical plant cells, in not having a recovery phase. A nerve fibre can propagate action potentials along its length repeatedly and at a very high frequency. The ability to do this depends upon the membrane reconstitution or recovery travelling along the nerve immediately behind the electrical depolarization which is one of the characteristics of the action potential. We can predict, on purely biological grounds, that if an egg responds to fertilization by propagating an action potential over its surface, the form of the electric change will be quite different from that observed in active nerve or muscle fibres, *because fertilization is, under ordinary conditions, irreversible.* The action potential would not, therefore, be expected to look like the normal one in Fig. 26*a*, but more like that shown in Fig. 26*b*, which has no recovery phase. Before considering the possibility of action potentials being propagated over egg surfaces at fertilization or activation and the claims which have been made to this effect, one should be clear as to what an action potential is, and how it is recorded. Two electrodes, connected to a voltmeter, are shown on the intact surface of a nerve fibre in Fig. 27*a*, with an action potential coming towards them from left to right. At this stage, the electrodes are equipotential as they are both on inactive parts of the nerve surface. When the action potential reaches electrode 1, Fig. 27*b*, a transient potential difference develops be-

tween the electrodes, because the 'breakdown' of the nerve membrane, which characterizes the action potential, causes electrode 1

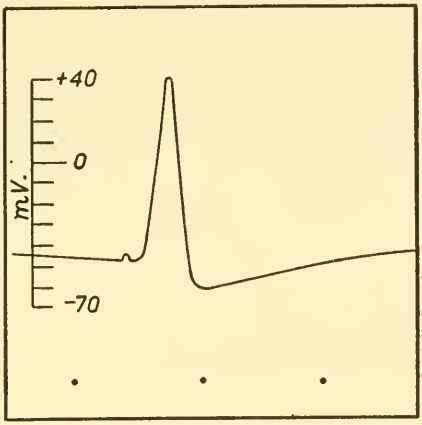


FIG. 26*a*.—Action potential recorded between inside and outside of squid giant axon. The vertical scale shows the potential in millivolts of one electrode inside the axon, relative to a second electrode in the external medium, assumed to be at zero potential (after Hodgkin & Huxley, 1945).

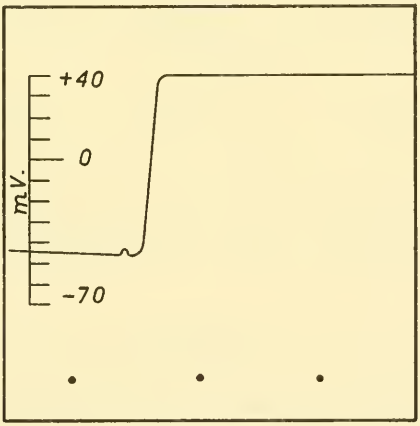


FIG. 26*b*.—The same action potential as in *a*, without any recovery phase. In both cases, the dots below the action potentials are $\frac{1}{500}$th of a second apart.

to be transiently connected to a 'sodium battery' (Fig. 28). In Fig. 27*c* the action potential is between the electrodes,* which

* The wave-length of the action potential is assumed to be short compared with the inter-electrode distance.

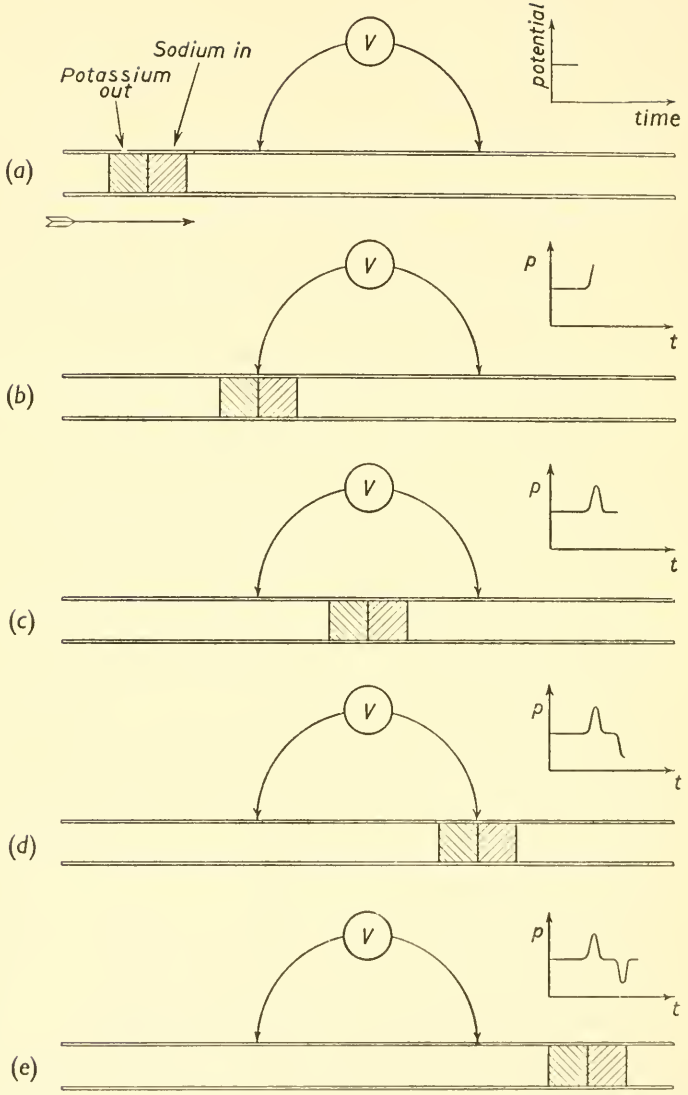


FIG. 27.—An action potential travelling along a nerve fibre from left to right, with two electrodes, connected to a voltmeter V, on the nerve surface.

are, therefore, equipotential again; while in Fig. 27*d* it has reached electrode 2, causing a second transient potential difference, which is the mirror image of Fig. 27*b*, between the electrodes. If the system under examination has not got a muscle attached to one end of it and cannot be repeatedly stimulated, a set-up of the type described above is necessary for a demonstration of conduction. Even then difficulties in interpretation may arise; the reader may like to consider the difference between a record of a propagated

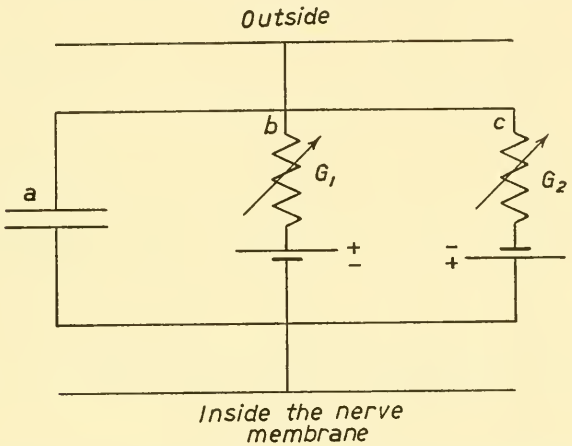


FIG. 28.—Electrical model of a nerve membrane: *a*, membrane capacitance; *b*, K^+ channel; *c*, Na^+ channel; G_1, membrane K conductance, 0·5 mmho/cm²; G_2, membrane Na conductance, 0·01 mmho/cm². The batteries are due to the unequal distribution of Na and K between the inside and outside of the nerve membrane. In the early phases of the action potential, G_2 rises (resistance falls), so that the recording system measures the potential due to the sodium battery. A 5-mV. change in the p.d. across the membrane causes an *e*-fold change in membrane conductance (adapted from Hodgkin & Huxley, 1952).

action potential with no recovery phase and a record of a non-propagated electrical change, with recovery, at one electrode.

If a resistance meter is substituted for the voltmeter in the above circuit, the resistance of a square centimetre of membrane can be shown to fall by a factor of 100 or more in the 'active' region, where there is a potential change of 100 mV. There is no capacitance change.

These are, very briefly, the electrical changes which constitute the action potential. Chemically, it consists of an influx of sodium ions during the rising phase, Fig. 26*a*, possibly due to the transient

removal of calcium from sites in the nerve membrane (Frankenhaeuser & Hodgkin, 1955), and an outflow of potassium during the falling or recovery phase.

What evidence is there that anything like the phenomena described in the preceding three paragraphs occurs at fertilization? Dorfman claimed in 1934 that there was a potential difference of 44 mV. between the inside and outside of the unfertilized frog's egg, the inside being negative, and that this potential difference was reversed at fertilization. As, however, the insertion of a needle or micro-electrode into an unfertilized frog's egg activates the egg parthenogenetically, the potential changes observed by Dorfman could have had little if anything to do with the early phases of fertilization. Apart from this, the reversal of p.d. took place one hour after insemination. In 1935, Hasama said he had observed electrical changes in the egg of *Hynobius nebulosus* (Schlegel) at fertilization. There is no reason to suppose that Hasama's published records are anything but random baseline fluctuations due to the measuring apparatus. A further claim that activation of the frog's egg, both by a spermatozoon and by a glass needle, is associated with electrical changes, was made in the same year by Péterfi & Rothschild (1935). No records and very few experimental details were published. When I systematically repeated these experiments a year or so later, I came to the conclusion that though the puncture of an unfertilized frog's egg was associated with electrical changes or signals which *might* be of biological origin, the form of the changes was unpredictable and the evidence that they were propagated over the egg surface inadequate. When, on the other hand, a frog's egg is fertilized, the evidence that bio-electric changes occur is more convincing, though such experiments present formidable technical difficulties; but there is no reason to suppose that such changes are propagated and to describe them as action potentials is wrong. Recent claims that 'action potentials' occur when sea-urchin eggs are fertilized (Scheer *et al.*, 1954) are open to the same criticisms as the experiments referred to above, which were done some twenty years ago. The most we can say is that when eggs are fertilized, potential changes of obscure origin are sometimes observed; but in the absence of further evidence, these should not be called action potentials, nor thought of as such.

There are several reasons for thinking that these potential

changes may not be of much importance in fertilization. In spite of the asymmetrical distribution of ions between the inside and outside of the sea-urchin egg (Table 20), which, unless the plasma

TABLE 20

Inorganic constituents of unfertilized eggs of Paracentrotus lividus *and of sea water (Rothschild & Barnes, 1953)*

	Eggs, mM	*Sea water, mM*
Sodium . . .	52	485
Potassium . . .	210	10
Calcium . . .	4	11
Magnesium . . .	11	55
Chloride . . .	80	566
Sulphate . . .	6	29
Total phosphorus . .	[2·1]	—

In the case of eggs, mM means millimoles per kilogram of water in the eggs (dry weight, 24%; density, 1·09); in the case of sea water, mM means millimoles per kilogram of water, chlorinity 19·37‰. The figure in square brackets for total phosphorus is in mg./ml. eggs.

membrane is impermeable to sodium and potassium, one would have expected to cause a potential difference across the egg surface, no such difference has been observed (Rothschild, 1938). In the 1938 experiments the terminal diameter of the electrode inserted into the egg was 2–10 μ. On modern standards an electrode of this size would be considered coarse and liable to tear the egg surface, with consequent short-circuiting and failure to record any potential difference. Suppose that there is a potential difference across the plasma membrane and that the insertion of a micro-pipette causes such short-circuiting as to make the potential difference so small as to be unmeasurable. The fact remains that eggs can be fertilized after the insertion of two such electrodes, which means that fertilization is not dependent upon the existence of a potential difference across the egg surface. It is far from clear how an action potential could be propagated over the egg surface, when, in the resting state, there is no potential difference across the membrane under consideration. The idea that no-one has ever got a micro-electrode, as opposed to an ultramicro-electrode, into a sea-urchin egg, and that the electrode merely causes an extended invagination of the plasma membrane, is scarcely tenable. It is, for example, possible to inject a live spermatozoon into the cytoplasm of a sea-urchin egg, though fertilization does not occur. Similarly, as is

well known, indicators and dyes have been repeatedly injected into sea-urchin eggs. I have confirmed the apparent lack of p.d. across the sea-urchin egg plasma membrane, using ultramicro-electrodes, and so have Scheer *et al.* (1954). Lundberg (1956) believes that when such electrodes are used, attempts at insertion are often unsuccessful because the electrode does cause an extended invagination of the plasma membrane. When, however, he did manage to effect contact with the egg cytoplasm, it appeared to be 5–10 mV. positive with respect to the external medium. In view of the size of liquid junction potentials in systems of this sort, a resting potential of 5–10 mV. is too close to zero to have much significance. Moreover, it is very difficult to see how the potential of the inside of the sea-urchin egg can be positive with respect to the outside, unless the plasma membrane is impermeable to potassium ions and permeable to sodium ions, the latter being actively pumped out.

To sum up this section: there is as yet no good evidence that the irregular potential changes observed in eggs at fertilization occur at the plasma membrane; nor that they are propagated over the egg surface and, therefore, connected with the block to polyspermy; nor that they are important in fertilization.

Membrane resistance. Very few estimates of egg membrane resistances have been made because of the technical difficulties inherent in such measurements on small spherical cells. The following calculation shows the origin of these difficulties, particularly if an a.c. method of measuring resistance is used, with external electrodes. Suppose that the membrane resistance is 500 ohm-cm.^{2} (Davson, 1951). The radius of a sea-urchin egg (*Psammechinus miliaris*) is 50μ, so that its surface area is about 3.10^{-4} cm^2. The actual resistance to be measured will, therefore, be of the order of 10^6 ohms, in comparison with which the resistance of the external medium is negligible. As a result, the only systematic measurements of egg membrane resistance are those of Cole & Guttman (1942), using the unfertilized egg of *Rana pipiens* Schreber. They obtained a value of 170 ohm-cm.^{2}, with an alternating current bridge method. If ultramicro-electrodes could be inserted into eggs without their tips being broken or clogged, the most satisfactory method of measuring egg membrane resistances would be by inserting two electrodes into the egg, flowing current across the plasma membrane using one of the internal electrodes,

and measuring the resultant ohmic drop of potential across the plasma membrane with the other internal electrode. Such an experiment would not be easy and might first be tried on unfertilized trout eggs in oil, before contact with tap water. This would avoid the complicating factor of the development of the chorion. Cole & Guttman calculated from Holzer's data (1933) that the trout egg membrane resistance was about 5,000 ohm-cm^2. However, they failed to notice that Holzer's experiments were done on trout eggs which had been cut in half. This treatment always kills the eggs and entirely destroys the resistive properties of the vitelline membrane; this hypothetical value for the trout egg membrane resistance should not, therefore, be accepted.

Lundberg (1956), using the 'ohmic drop' method with two

TABLE 21

Egg membrane capacitance, c_m, and egg cytoplasm resistivity, r_2

Species	c_m, $\mu F/cm^2$		r_2, *ohm-cm*		*Method*	*Reference*
	U	F	U	F		
A. punctulata	0·86	3·3	105	133	a.c. (intact eggs)	Cole & Spencer, 1938
T. ventricosus	0·87	2·0	203	349	,,	Cole, 1935
A. forbesi	1·10		136–225		,,	Cole & Cole, 1936*a*
R. pipiens	2·0		570		,,	Cole & Guttman, 1942
S. trutta	0·57	0·58	202 *	159 *	,,	Rothschild, 1946
S. trutta			90 *		a.c., directly on contents	Gray, 1932

* These measurements were on the inner contents of the egg, not the egg cytoplasm (see text).
U, unfertilized; F, fertilized.

ultramicro-electrodes inside the egg (*Psammechinus miliaris*), managed to make a satisfactory measurement on one egg and obtained the value 2,200 ohm-cm^2 for its membrane resistance. In his other measurements, great difficulties were experienced, as mentioned earlier, in establishing electrical contact between the electrodes and the egg interior.

Membrane capacitance. The most important work on this subject is that of Cole and his co-workers. His results on egg membrane capacitance and egg cytoplasm resistivity, together with a few others, are given in Table 21. Cole made two important discoveries in his capacitance measurements on eggs. First, that there is a marked increase in membrane capacitance when the eggs of *Arbacia punctulata* and of *Tripneustes ventricosus* (Lamarck) are fertilized (about 400% and 240%). This has been confirmed by Iida (1943*a*, *b*), using the eggs of *Pseudocentrotus depressus* and

Hemicentrotus pulcherrimus. The approximate time course of the capacitance change is shown in Fig. 29. The reader should beware of a somewhat confusing interpretation of this phenomenon, involving a large capacitance in the fertilization membrane, put forward at one time by Cole & Cole (1936*b*). This interpretation is most unlikely to be correct, as Cole & Spencer pointed out in 1938. The change in capacitance at fertilization is real and reflects the structural changes in the cell surface which are known

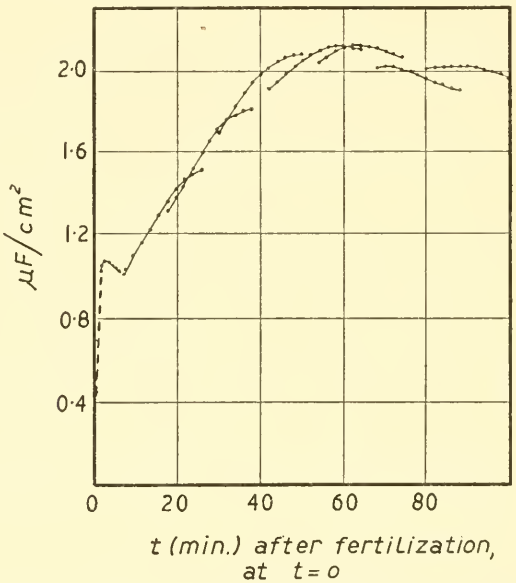


FIG. 29.—Changes in membrane capacitance of eggs of *Pseudocentrotus depressus* following fertilization. A set of points connected by a line refers to consecutive readings on a sub-sample containing lightly centrifuged eggs, removed from one parent sample. Cleavage at 80–100 min. in parent sample. T° C., 17·9–18·5. Adapted from Iida (1943*a*).

to take place at that time. Apart from what might be called chemical changes, the cell surface becomes thinner at fertilization, because of the elevation of the vitelline (= fertilization) membrane. This reduction in thickness is consistent with the observed increase in capacitance, for the following reason: the capacitance of a parallel plate condenser is given by the equation

$$C = \varepsilon A/4\pi l \quad . \quad . \quad . \quad (3)$$

where ε = dielectric constant of the medium between the plates,

A = cross-sectional area of plates, and l = distance between the plates. If l becomes smaller, i.e., the condenser becomes thinner, the capacitance becomes larger. Chemical changes in the cell surface may, of course, also be responsible for the observed change, but with our present exiguous knowledge of the chemistry of the cell surface, it is difficult even to speculate about the nature of changes which might cause an increase in capacitance.

It would be of great interest to try to find out at what rate this capacitance increase, which presumably starts at the site of sperm attachment, is propagated over the egg surface. Such an experiment would again probably involve difficult experiments with ultramicro-electrodes.

The second discovery that Cole (1935) made in this field was that the membrane capacitance of the eggs of *Tripneustes ventricosus* is inversely proportional to the surface area of the egg, when this is varied by diluting the sea water around the eggs with distilled water. This also has been confirmed by Iida (1943*c*), using the eggs of *Pseudocentrotus depressus*, in which the changes in membrane capacitance associated with alterations in the hypotonicity of the sea water were found to be reasonably reversible. This shows that the unexpected sense of the capacitance change is not due to irreversible injury of the cortex following stretching. Iida's results are given in Fig. 30. Unless an egg in hypotonic sea water continually synthesizes new membrane material to maintain its normal thickness (curve B, Fig. 30), a most improbable situation, the membrane capacitance should increase (curve A, Fig.30), not decrease, when the egg swells. The reasons are clear, as before, from a consideration of Equ. (3). These observations may well have revealed a fundamental, but paradoxical property, of cell membranes in general, quite apart from those of sea-urchin eggs. They merit further investigation, particularly in conjunction with the Elastimeter experiments of Mitchison & Swann (1954*a*), discussed in chapter 8. When considering the interpretation of these capacitance changes, Iida (1943*c*, p. 171) says: 'If an assumption is made that the membrane is of a mosaic structure with two intermingling areas, of which one is "effective" and the other is "ineffective" in manifesting measureable capacitance, and if the latter area alone is extensible on mechanical stretching, the capacitance will vary in a manner represented by C. A scheme like this appears to be a little too artificial, but it may not be altogether

physically implausible.' * An alternative interpretation of these 'anomalous' capacitance changes is that in the unswollen condition, the sea-urchin egg membrane is folded, on a sub-microscopic scale, and that when the egg swells, the membrane unfolds. Under these conditions, calculation of the membrane capacitance per unit area for the unswollen egg will produce too high a value, but the calculation for the swollen egg will be more accurate. If, in fact, the membrane is a 'reasonable' one (curve A, Fig. 30), it should be

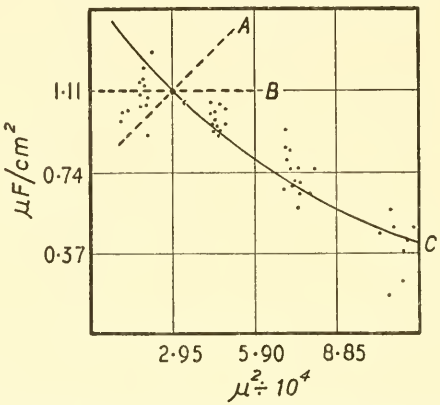


FIG. 30.—Change in egg membrane capacitance per unit area with surface area of egg, the latter being varied by immersing the eggs in sea water of different degrees of hypotonicity. Fertilized eggs of *Pseudocentrotus depressus* were used. *A*, theoretical curve showing expected behaviour of the membrane capacitance when the egg swells; *B*, theoretical curve if the membrane thickness remains constant while the egg swells; *C*, variation in membrane capacitance as an inverse function of the surface area. Adapted from Iida (1943*c*).

possible to make an estimate of the minimum degree of folding from capacitance measurements in normal and hypotonic sea water.

Cytoplasmic resistivity. Table 21 gives the results of experiments in this field. The differences between unfertilized and fertilized eggs may or may not be significant; in any case, the most important changes which occur in the early phases of fertilization, with which we are concerned in this chapter, take place at the cell surface †

* Unfortunately, a further paper dealing with the membrane capacitance of sea-urchin eggs, in Japanese, by Iida (1949) *Zool. Mag.* (Dobutsugaku Zasshi), **58**, 122–125, is not available in the United Kingdom.

† K. Dan (1947) reported a small change in ζ potential after fertilization of the eggs of *Pseudocentrotus depressus* and *Anthocidaris crassispina*; but the change is too small to be of much interest.

and not in the cytoplasm. Later, chemical changes in the cytoplasm become of profound importance, but it is doubtful whether or not straightforward measurements of cytoplasmic conductivity will produce information of great interest. In the case of the column headed r_2 in Table 21, the values for the trout egg refer to the globulin-containing solution *within* the vitelline membrane and not to the cytoplasm, as in the other cases mentioned in this Table. There is no cytoplasm to speak of in the unfertilized trout egg.

To summarize the contents of this chapter, measurements of bio-electric phenomena at fertilization have so far revealed little of importance except for the increase in membrane capacitance when sea-urchin eggs are fertilized. The main reason for this unsatisfactory state of affairs is the refractory nature of the biological material and the associated technical difficulties. The reader should consult Cole's papers for further details of experiments involving alternating current measurements, while the theory of such measurements when applied to eggs, and such questions as the meaning of polarization capacitances, characteristic frequencies, and the frequency-dependence of membrane capacitances are summarized by Rothschild (1946).

Note. Since this chapter was written, the results of new experiments on the bioelectric properties of eggs have been published. Although they do not in general affect my conclusions, they are sufficiently important to deserve special mention. Grundfest *et al.* (1955)* and Tyler *et al.* (1955)† observed a potential difference of 30–60 mV. between the inside and the outside of the starfish egg, the inside being negative. The p.d. could be reversibly reduced to zero by increasing the K content of the surrounding sea water. The membrane resistance and capacitance were, respectively, 2,000–3,000 ohm-cm^2 and 0·5–1·0 μF/cm^2. The p.d. decreased transiently at about the time of fertilization and, within a minute of insemination, increased to 10–15 mV. above the "resting potential".

* Grundfest, H., Kao, C. Y., Monroy, A. & Tyler, A. (1955) *Biol. Bull., Wood's Hole*, **109**, 346.

† Tyler, A., Monroy, A., Kao, C. Y. & Grundfest, H. (1955) *Biol. Bull., Wood's Hole*, **109**, 352–353.

CHAPTER 11

SPECIFICITY

THE reactions between the gametes exhibit a high, but not total, degree of specificity, whether the reaction is fertilization or the agglutination of spermatozoa by egg water. Quite apart from environmental barriers in nature, we can, for example, be virtually certain that bull spermatozoa will be unable to fertilize or even activate a rabbit egg. In modern language, p, the probability of a successful sperm-egg collision (i.e. one which achieves fertilization), or α, the sperm-egg interaction rate, will be extremely low in such a case; p will not be zero and might be made appreciably greater than zero by appropriate treatment of the egg. One such treatment, which has been extensively but empirically used in effecting cross-fertilization, is to have an abnormally large number of heterologous spermatozoa round the eggs in question. The probabilistic analysis of fertilization, discussed in chapter 9, Polyspermy, explains why, but not how, an increase in sperm density improves the chances of cross-fertilization. Equ. (2) in chapter 9 can be written in the approximate form

$$u = \exp(-\pi a^2 n \bar{c} p t) \quad . \quad . \quad (4)$$

where u = proportion of unfertilized eggs in a suspension allowed to interact with spermatozoa, density n, for a known time t; a = egg radius; $\bar{c}$ = mean speed of the spermatozoa; and p = probability of a successful collision.

We can make u smaller, i.e. increase the number of fertilized eggs, by any of the following operations:

(1) *Increase* $\bar{c}$. In general, this is extremely difficult, if not impossible, to do, though it is conceivable that Loeb's method of improving cross-fertilization, which involved making the sea water more alkaline (1903), worked partly in this way. Sea-urchin spermatozoa sometimes become more active in alkaline sea water.

(2) *Increase t.* Although this can always be done to a certain extent, the fertilizing life span of spermatozoa is limited, and so is the life of the unfertilized egg. In any case, the technique of known sperm-egg interaction times was only developed in 1950 and, at the

time of writing this book, the method has not been applied to cross-fertilization experiments, the unfertilized eggs simply being left in contact with the heterologous spermatozoa for an unknown and indefinite time.

(3) *Increase p.* p can sometimes be increased by removal of egg jelly (Harding & Harding, 1952*a*), by pre-treatment of the gametes with glycine + egg water or sodium periodate + egg water (Harding & Harding, 1952*b*), or by interfering with the vitelline membrane of unfertilized eggs through treatment with such agents as trypsin (Hultin, 1948). Trypsin also induces polyspermy in homologous fertilization, as Hagström & Hagström (1954*c*) have recently shown. One might envisage the surface of an unfertilized egg as a three-dimensional jig-saw puzzle, made of rubber and containing very weak magnets. Of course, the magnets are, in reality, van der Waals' forces which are inversely proportional to the seventh power of the distances between the atoms involved, hydrogen bonds and attractions between oppositely charged groups (Pauling *et al.*, 1943). The application of trypsin could be likened to a bunsen burner turned on to the jig-saw puzzle. Some of the protuberances on the surface will be melted, decreasing the preciseness of fit with complementary structures on the head of the homologous spermatozoon, but permitting a reaction to occur with less precisely complementary structures on the heads of some heterologous spermatozoa. An alternative interpretation of the effect of trypsin is, however, possible—that it exposes more or 'deeper' combining groups. Incomplete antibody, for example, combines with antigens on red blood cells, though no agglutination takes place. But if the red blood cells are pre-treated with trypsin, washed, and then subjected to incomplete antibody, agglutination occurs (Coombs, 1954). This suggests that, so far from blunting combining groups, treatment with trypsin sharpens or exposes them. A similar conclusion might be reached from the work of Coffin & Pickles (1953), who found that periodate destroyed the D Rh antigen and that subsequent treatment with trypsin brought back the property of agglutination. The procedure could be repeated, as if combining sites were obliterated by periodate, but that new ones were exposed by a second treatment with trypsin.

Runnström *et al.* (1944*b*) found no improvement in cross-fertilizations between *Psammechinus miliaris* and *Echinocardium cordatum* after pre-treating the unfertilized eggs with trypsin,

while Hultin (1948) had the same experience with other sea-urchins.

(4) *Increase n.* This is the classical way of achieving cross-fertilization. An increase in sperm density involves an increase in the number of sperm-egg collisions as the latter, Z, is equal to $\pi a^2 n\bar{c}$. For any p, however low, the more collisions there are, the greater the chance that an egg will be fertilized.* The most important investigation of cross-fertilization in which n was controlled was made by Tyler (1949), though Fuchs (1914–1915) also

TABLE 22

Comparison between cross-fertilization and cross-agglutination

The upper figures in each pair refer to the dilution of a standard sperm suspension necessary to achieve 2% fertilization (e.g., 3000 means 1/3000). The lower figures in each pair are the highest dilutions of fertilizin solution at which visible agglutination occurred.

Eggs or fertilizin of	*Spermatozoa of*			
	S. purpuratus	S. franciscanus	L. pictus	D. excentricus
S. purpuratus	3,000 512	3 8	1 64	5 4
S. franciscanus	1 —	600 512	1 4	2½ —
L. pictus	2 64	4 32	850 64	2½ 8
D. excentricus	1½ 4	40 2	2 1	4,400 128

realized the importance of controlling n, in his studies on self-sterility in *Ciona intestinalis*. Tyler's results are reproduced in Table 22. The experiments were done for a particular reason, to compare cross-fertilization and cross-agglutination; the technique of known sperm-egg interaction times had not been developed at that time. Tyler's results are interesting as they show that the correspondence between the degree of cross-fertilizability and cross-agglutination is not particularly marked. This means that there is more specificity in fertilization than in the fertilizin–antifertilizin reaction, or that fertilization is not ex-

* This is not the same proposition as the familiar but fallacious one about red being 'bound' to turn up after a run of 20 blacks, at roulette.

clusively determined by the fertilizin–antifertilizin reaction. On the other hand, Table 22 shows that in general, cross-fertilization implies cross-agglutination. Even in the apparently exceptional cases of eggs or fertilizin from *Strongylocentrotus franciscanus* (A. Agassiz) or spermatozoa from *Strongylocentrotus purpuratus* and *Dendraster excentricus* (Eschscholtz), Tyler has shown that spermatozoa of the latter echinoderms do combine with fertilizin of the former.

The partially successful interphyletic crosses which have been achieved, such as *Strongylocentrotus* ♀ × *Mytilus* ♂ (Kupelwieser, 1909), raise a number of interesting but difficult problems. p will clearly be very low in such cases; but there is no particular reason why a successful hit should not be achieved from time to time, even if subsequent development is gynogenetic. Kupelwieser's experiments do not exclude an alternative possibility: that such crosses can be achieved without specific adhesion between the surfaces of the egg and the spermatozoon, but as a result of the action of a non-specific lysin or detergent-like compound, diffusing out of the sperm head and softening up the egg cortex so that the sperm can be readily engulfed. F. R. Lillie (1919) favoured this interpretation of Kupelwieser's results, and there have been references elsewhere in this book to the possibility of misinterpreting experiments involving insemination with very high sperm concentrations, because of the possibility of non-specific effects due to sperm lysins or A.III. But even if, intuitively, we do not like the idea of there being a sufficient degree of complementariness between the surface of eggs and spermatozoa of different phyla to permit activation, we must still admit that the possibility exists, first because the 'fit' does not have to be complete (see later in this chapter), and secondly, because the reaction is essentially a probabilistic one, in which p does not equal zero. Whatever one's intuitive feelings may be, cross reactions do occur: a classical example is the Weil Felix Reaction, in which *B. proteus* is agglutinated by serum from patients with typhus!

Apart from certain examples and analogies, what has been said above about specificity is mainly empirical, or analytical, in the sense of being based on the laws of probability and the kinetic theory of gases. When we come to examine the 'how' part of the problem, almost complete ignorance prevails, in spite of the immense number of cross-fertilization experiments which have been done during

this century. Even in 1900, for example, Vernon could report twenty-nine different and successful echinoderm crosses; but though these and subsequent cross-fertilization experiments are interesting from an embryological and genetical point of view, they do not help in gaining an understanding of specificity and self-sterility. In general terms the specific reaction between a spermatozoon and an egg is due to complementariness of surface structure which allows close contact to occur over an area which, by analogy with serological reactions, might be of the order of 100 $Å^2$; this enables the weak forces of intermolecular interaction to combine to produce a strong union. The fit of an antibody to an antigen is close, the complementariness in structure being such that an increase of 0·8 Å in the size of one atom in a group can cause steric hindrance. On the other hand, cross-fertilization does not imply that the heterologous spermatozoa in question have surface combining configurations which are identical with those on homologous spermatozoa. For example, in antigenic proteins containing azobenzene arsonic groups, the substitution of a methyl group by a chlorine or bromine atom causes little change in serological properties, because the van der Waals' radii of the methyl group, chlorine and bromine are, respectively, 1·9, 1·81 and 1·95 Å. We can imagine that different groups in particular regions of the folded polypeptide chains, which probably constitute the protein parts of the reacting molecules, sometimes have sufficiently similar van der Waals' radii in different species to allow an unexpected cross-reaction to take place. The 'rubberiness' of the fit, referred to in the jig-saw puzzle analogy, is reflected in the behaviour of anti-*p*-(*p*-azobenzene-azo) benzene arsonic acid antiserum, in which a radial dilation of about 1·5 Å is possible without interference with the reaction.

To sum up, the only advances that have been made in the study of specificity in fertilization since F. R. Lillie wrote a chapter on this subject, which all students of fertilization should read, in *Problems of Fertilization*, are: (1) more information about the immunological nature of the reactions between the gametes, almost entirely due to the work of Tyler; (2) more information about the nature of immunological reactions in general, much of which is due to Landsteiner and Pauling; and, possibly, (3) the development of techniques for the quantitative study of interspecific and intergeneric cross-fertilization.

CHAPTER 12

CONCLUSION

In my Preface I expressed a hope that this book would provoke further experiments. The question is, what experiments? Prophecies are always dangerous and usually wrong; but for what it is worth, I believe that the following subjects would repay further investigation:

(1) Morphology of pronuclear movements.

(2) Sub-microscopic morphology of spermatozoa in sea water and egg water.

(3) Physiology of frog's egg jelly.

(4) Turning mechanisms in plant spermatozoa.

(5) Structure-action relationships in the chemotaxis of plant spermatozoa.

(6) Variations in the receptivity of different parts of the egg surface.

(7) Oxidative carbohydrate breakdown in eggs.

(8) DNA content of eggs, spermatozoa and pronuclei.

(9) Partial fertilization, using 'cylindrical' eggs.

(10) Irritability of the *zona pellucida*.

(11) K and Na fluxes across egg surface before and after activation.

(12) Conduction velocity of capacitance change at fertilization.

(13) Membrane resistance before and after activation. This may be linked with (11).

(14) Effects of periodate and trypsin on heterologous fertilization.

(15) The morphology, physiology and biochemistry of fertilization and parthenogenetic activation *in any eggs other than those of echinoderms*. Japanese biologists have realised the importance of this subject and in their hands it is beginning to pay dividends. Novikoff's experiments, on the escape of

cortical granules from the fertilized egg of *Sabellaria alveolata*, should cause those who work exclusively on echinoderm eggs some anxiety.

Other lists could, of course, be made. The reasons why my list contains what it does are to be found, I hope, in this book.

REFERENCES AND AUTHOR INDEX

The numbers in italics after a reference refer to the pages on which this work of the author is mentioned, for example:

ENDO, Y. (1952) *Exp. Cell Res.*, **3**, 406–418. *6, 9, 10, 98, 107.*

means that Endo's paper is mentioned on pages 6, 9, 10, 98 and 107.

ABELSON, P. H. (1947) *Biol. Bull., Wood's Hole*, **93**, 203. *81.*
ABELSON, P. H. (1948) *Biol. Bull., Wood's Hole*, **95**, 262. *81.*
ABRAMS, R. (1951) *Exp. Cell Res.*, **2**, 235–242. *84.*
ALFERT, M. & SWIFT, H. (1953) *Exp. Cell Res.*, **5**, 455–460. *85.*
ALLEN, R. D. (1953) *Biol. Bull., Wood's Hole*, **105**, 213–239. *119.*
ALLEN, R. D. (1954) *Exp. Cell Res.*, **6**, 403–424. *6, 15, 104, 119, 121.*
AMOROSO, E. C. & PARKES, A. S. (1947) *Proc. roy. Soc. B*, **134**, 57–78. *119.*
ASHBEL, R. (1929) *Boll. Soc. ital. Biol. sper.*, **4**, 492–493. *64, 65.*
AUSTIN, C. R. (1951*a*) *J.R. micr. Soc.*, **71**, 295–306. *18.*
AUSTIN, C. R. (1951*b*) *Aust. J. sci. Res., B*, **4**, 581–596. *13, 114.*
AUSTIN, C. R. (1953) *Aust. vet. J.*, **29**, 191–198. *12.*
AUSTIN, C. R. & BRADEN, A. W. H. (1953) *Aust. J. biol. Sci.*, **6**, 674–692. *106.*
AUSTIN, C. R. & BRADEN, A. W. H. (1954*a*) *Aust. J. biol. Sci.*, **7**, 179–194. *115.*
AUSTIN, C. R. & BRADEN, A. W. H. (1954*b*) *Aust. J. biol. Sci.*, **7**, 195–210. *115.*
BALLENTINE, R. (1940) *J. cell. comp. Physiol.*, **15**, 217–232. *60, 61.*
BARRON, E. S. G. (1932) *Biol. Bull., Wood's Hole*, **62**, 42–45. *61.*
BATAILLON, E. (1909) *Arch. EntwMech. Org.*, **28**, 43–48. *1.*
BEAMENT, J. (1946) *Proc. roy. Soc. B*, **133**, 407–418. *4.*
BERG, W. E. (1950*a*) *Biol. Bull., Wood's Hole*, **98**, 128–138. *37.*
BERG, W. E. (1950*b*) *Proc. Soc. exp. Biol., N.Y.*, **75**, 30–32. *87.*
BERNSTEIN, M. H. (1949) *Biol. Bull., Wood's Hole*, **97**, 255. *84.*
BIAŁASZEWICZ, K. (1912) *Arch. EntwMech. Org.*, **34**, 489–540. *11.*
BIELIG, H.-J. & DOHRN, P. (1950) *Z. Naturf.*, **5b**, 316–338. *33.*
BISHOP, D. W. (1951) *Biol. Bull. Wood's Hole*, **101**, 215–216. *26.*
de BLAINVILLE, H. M. D. (1825) *Dictionnaire des Sciences Naturelles*, **37**, F. G. Levrault, Strasbourg & Paris. *154.*
de BLAINVILLE, H. M. D. (1834) *Manuel d'Actinologie*, F. G. Levrault, Strasbourg & Paris. *154.*
BLANDAU, R. J. & ODOR, D. L. (1952) *Fertility and Sterility*, **3**, 13–26. *12.*
BLOCH, F. (1935) *Trav. Sta. zool. Wimereux*, **12**, 183–279. *v.*
BLOUNT, M. (1909) *J. Morph.*, **20**, 1–64. *103.*
BLYTH, J. S. S. (1954) *Poult. Sci.*, **33**, 310–315. *105.*
BOLOGNARI, A. (1952) *Arch. Sci. biol.*, **36**, 40–47. *78, 90.*
BONNEVIE, K. (1907) *Jena Z. Naturw.*, **35**, 567–598. *103.*
BOREI, H. (1933) *Z. vergl. Physiol.*, **20**, 258–266. *64, 65.*
BOREI, H. (1948) *Biol. Bull., Wood's Hole*, **95**, 124–150. *57, 61.*
BOREI, H. (1949) *Biol. Bull., Wood's Hole*, **96**, 117–122. *57, 59.*
BOREI, H. (1951) *Acta chem. scand.*, **4**, 1607–1608. *67.*
BOVERI, T. (1907) *Jena Z. Naturw.*, **36**, 1–292. *105.*
BOYD, M. (1928) *Biol. Bull., Wood's Hole*, **55**, 92–100. *61, 63.*
BRACHET, J. (1933) *Arch. Biol., Paris*, **44**, 519–576. *84.*
BRACHET, J. (1934*a*) *Arch. Biol., Paris*, **45**, 611–727. *65.*
BRACHET, J. (1934*b*) *Arch. Biol., Paris*, **46**, 1–24. *56, 122.*

Brachet, J. (1950) *Chemical Embryology*, Interscience Publishers Inc., New York. *62, 65, 74, 75.*
Brachet, J. (1954) *Arch. Biol., Paris*, **65**, 1–72. *v.*
Braden, A. W. H., Austin, C. R. & David, H. A. (1954) *Aust. J. biol. Sci.*, **7**, 391–409. *12, 106, 114.*
Bretschneider, L. H. (1948) *Proc. kon. Ned. Akad. Wet.*, **51**, 3–7. *103.*
Brock, N., Druckrey, H. & Herken, H. (1938) *Arch. exp. Path. Pharmak*, **188**, 451–464. *61.*
Brookbank, J. W. & Whiteley, A. H. (1954) *Biol. Bull., Wood's Hole*, **107**, 57–63. *89.*
Brooks, S. C. & Chambers, E. L. (1948) *Biol. Bull., Wood's Hole*, **95**, 262–263. *81.*
Brooks, S. C. & Chambers, E. L. (1954) *Biol. Bull., Wood's Hole*, **106**, 279–296. *82.*
Bruchmann, H. (1909) *Flora, Jena*, **99**, 193–202. *50.*
Burdon-Jones, C. (1951) *J. Mar. biol. Ass. U.K.*, **29**, 625–638. *21.*
de Burgh, P. M., Sanger, R. A. & Walsh, R. J. (1946) *Aust. J. exp. Biol. med. Sci.*, **24**, 293–300. *29.*
Byers, H. L. (1951) *Biol. Bull., Wood's Hole*, **101**, 218. *24.*
Carter, G. S. (1931) *J. exp. Biol.*, **8**, 176–201. *33.*
Cennamo, C. & Montella, S. (1947) *Experientia*, **3**, 415–416. *66.*
Chambers, E. L. (1939) *J. exp. Biol.*, **16**, 409–424. *15–18.*
Chambers, E. L. (1949) *Biol. Bull., Wood's Hole*, **97**, 251–252. *83.*
Chambers, E. L. & Mende, T. (1953*a*) *Arch. Biochem. Biophys.*, **44**, 46–56. *82.*
Chambers, E. L. & Mende, T. (1953*b*) *Exp. Cell Res.*, **5**, 508–519. *82.*
Chambers, E. L. & White, W. E. (1949) *Biol. Bull., Wood's Hole*, **97**, 225–226. *82.*
Chambers, E. L. & White, W. E. (1954) *Biol. Bull., Wood's Hole*, **106**, 297–307. *82.*
Chambers, E. L., White, W., Jeung, N. & Brooks, S. C. (1948) *Biol. Bull., Wood's Hole*, **95**, 252–253. *83.*
Chambers, R. (1921) *Biol. Bull., Wood's Hole*, **41**, 318–350. *20.*
Chambers, R. (1923) *J. gen. Physiol.*, **5**, 821–829. *7.*
Chambers, R. (1942) *J. cell. comp. Physiol.*, **19**, 145–150. *11.*
Chang, M. C. & Pincus, G. (1953) *Science*, **117**, 274–276. *55.*
Churney, L. & Moser, F. (1940) *Physiol. Zool.*, **13**, 212–217. *99.*
Clark, J. M. (1936) *Biol. Bull., Wood's Hole*, **70**, 361–384. *116.*
Cleland, K. W. (1950*a*) *Proc. Linn. Soc. N.S.W.*, **75**, 282–295. *56, 59–61, 64, 65, 69.*
Cleland, K. W. (1950*b*) *Proc. Linn. Soc. N.S.W.*, **75**, 296–319. *66, 67, 69, 77, 78.*
Cleland, K. W. & Rothschild, Lord (1952*a*) *J. exp. Biol.*, **29**, 285–294. *70, 71.*
Cleland, K. W. & Rothschild, Lord (1952*b*) *J. exp. Biol.*, **29**, 416–428. *71, 78.*
Coffin, S. F. & Pickles, M. M. (1953) *J. Immunol.*, **71**, 177–182. *137.*
Cohen, A. & Berrill, N. J. (1936) *Biol. Bull., Wood's Hole*, **70**, 78–88. *19.*
Cole, K. S. (1932) *J. cell. comp. Physiol.*, **1**, 1–9. *92.*
Cole, K. S. (1935) *J. gen. Physiol.*, **18**, 877–887. *131, 133.*
Cole, K. S. & Cole, R. H. (1936*a*) *J. gen. Physiol.*, **19**, 609–623. *131.*
Cole, K. S. & Cole, R. H. (1936*b*) *J. gen. Physiol.*, **19**, 625–632. *132.*
Cole, K. S. & Guttman, R. M. (1942) *J. gen. Physiol.*, **25**, 765–775. *130, 131.*
Cole, K. S. & Spencer, J. M. (1938) *J. gen. Physiol.*, **21**, 583–590. *131, 132.*
Colwin, A. L. & Colwin, L. H. (1953) *J. Morph.*, **92**, 401–454. *20.*

COLWIN, L. H. & COLWIN, A. L. (1949) *Biol. Bull., Wood's Hole*, **97**, 237. *8.*
CONKLIN, E. G. (1905) *J. Acad. nat. Sci. Philad.*, **13**, 1–119. *16.*
CONNORS, W. M. & SCHEER, B. T. (1947) *J. cell. comp. Physiol.*, **30**, 271–283. *70, 82.*
COOK, A. H., ELVIDGE, J. A. & HEILBRON, Sir I. (1948) *Proc. roy. Soc. B*, **135**, 293–301. *47.*
COOK, A. H. & ELVIDGE, J. A. (1951) *Proc. roy. Soc. B*, **138**, 97–114. *47.*
COOMBS, R. R. A. (1954) *Schweiz. Z. allg. Path.*, **17**, 424–439. *137.*
COSTELLO, D. (1938) *Anat. Rec.*, **72**, Abstracts p. 112. *85.*
CRAGG, F. W. (1920) *Indian J. med. Res.*, **8**, 32–79. *4.*
CRANE, R. K. & KELTCH, A. K. (1949) *J. gen. Physiol.*, **32**, 503–509. *78.*
DALCQ, A. (1924) *Arch. Biol., Paris*, **34**, 507–674. *98.*
DALCQ, A. (1952) *Initiation à l'embryologie générale.* Masson & Cie, Paris. *2, 3.*
DALCQ, A., PASTEELS, J. & BRACHET, J. (1936) *Mém. Mus. Hist. nat. Belg.*, 2nd ser. **3**, 881–912. *98.*
DALY, J. M. (1954) *Arch. Biochem. Biophys.*, **51**, 24–29. *68.*
DAN, J. C. (1950*a*) *Biol. Bull., Wood's Hole*, **99**, 399–411. *5, 7, 12.*
DAN, J. C. (1950*b*) *Biol. Bull., Wood's Hole*, **99**, 412–415. *13, 14, 39.*
DAN, J. C. (1952) *Biol. Bull., Wood's Hole*, **103**, 54–66. *30.*
DAN, J. C. (1954) *Biol. Bull., Wood's Hole*, **107**, 203–218. *8.*
DAN, J. C. (1955) *Biol. Bull., Wood's Hole*, **107**, 335–349. *8, 30.*
DAN, K. (1947) *Biol. Bull., Wood's Hole*, **93**, 259–266. *134.*
DAVSON, H. (1951) *A Textbook of General Physiology.* J. & A. Churchill Ltd., London. *130.*
DONNELLON, J. A. (1938) *Physiol. Zool.*, **11**, 389–397. *99.*
DORFMAN, W. A. (1934) *Protoplasma*, **21**, 245–257. *128.*
DOYLE, W. L. (1938) *J. cell. comp. Physiol.*, **11**, 291–300. *87.*
ENDO, Y. (1952) *Exp. Cell Res.*, **3**, 406–418. *6, 9, 10, 98, 107.*
EPHRUSSI, B. (1933) *Arch. Biol., Paris*, **44**, 1–148. *65.*
FANKHAUSER, G. (1925) *Arch. EntwMech. Org.*, **105**, 501–580. *103, 104.*
FANKHAUSER, G. (1932) *J. exp. Zool.*, **62**, 185–235. *103, 104.*
FANKHAUSER, G. (1934) *J. exp. Zool.*, **67**, 159–215. *103, 104.*
FANKHAUSER, G. (1948) *Ann. N.Y. Acad. Sci.*, **49**, 684–708. *103, 104.*
FANKHAUSER, G. & MOORE, C. (1941) *J. Morph.*, **68**, 347–385. *103.*
FAURÉ-FRÉMIET, E. (1913) *Arch. mikr. Anat.*, **15**, 437–757. *20.*
FAURÉ-FRÉMIET, E. (1922) *C.R. Soc. Biol., Paris*, **86**, 20–23. *61.*
FENN, W. O. & COBB, D. M. (1932) *Amer. J. Physiol.*, **102**, 379–401. *67.*
FLOREY, Sir H. (1955) *Proc. roy. Soc. B*, **143**, 147–158. *96.*
FOL, H. (1877) *Arch. Zool. exp. gén.*, **6**, 145–169. *7.*
FOL, H. (1879) *Mém. Soc. Phys. Genève*, **26**, 89–250. *11, 18.*
FOLKES, B. F., GRANT, R. A. & JONES, J. K. N. (1950) *J. chem. Soc.*, Part III, 2136–2140. *38.*
FÖRSTER, H. VON & WIESE, L. (1954) *Z. Naturf.*, **9b**, 470–471. *54.*
FOX, H. M. (1921) *J. gen. Physiol.*, **3**, 501–511. *47.*
FRANK, J. A. (1939) *Biol. Bull., Wood's Hole*, **76**, 190–216. *21, 34, 37.*
FRANKENHAEUSER, B. & HODGKIN, A. L. (1955) *J. Physiol.*, **128**, 40P–41P. *128.*
FRUTON, J. S. & SIMMONDS, S. (1953) *General Biochemistry*, John Wiley & Sons, Inc., New York. *v.*
FUCHS, H. M. (1914) *Arch. EntwMech. Org.*, **40**, 157–252. *138.*
FUCHS, H. M. (1915) *J. Genet.*, **4**, 215–301. *138.*
GALTSOFF, P. S. (1940) *Biol. Bull., Wood's Hole*, **78**, 117. *21.*
GATENBY, J. B. & HILL, J. P. (1924) *Quart. J. micr. Sci.*, **68**, 229–238. *106.*
GLASER, O. (1913) *Science*, **38**, 446–450. *20.*
GLASER, O. (1914) *Biol. Bull., Wood's Hole*, **26**, 84–91. *20.*

GLASER, O. (1921) *Amer. Nat.*, **55**, 368. *23, 38.*
GLASER, O. (1924) *Biol. Bull., Wood's Hole*, **47**, 274–283. *20.*
GODLEWSKI, Jun., E. (1912) *Arch. EntwMech. Org.*, **33**, 196–254. *2.*
GOLDINGER, J. M. & BARRON, E. S. G. (1946) *J. gen. Physiol.*, **30**, 73–82. *78, 84.*
GOLDSCHMIDT, R. & KATSUKI, K. (1931) *Biol. Zbl.*, **51**, 58–74. *105.*
GRAY, J. (1920) *Proc. roy. Soc. B*, **91**, 147–157. *24.*
GRAY, J. (1927) *Brit. J. exp. Biol.*, **5**, 102–111. *11.*
GRAY, J. (1928) *Brit. J. exp. Biol.*, **5**, 362–365. *32.*
GRAY, J. (1931) *Experimental Cytology*, University Press, Cambridge. *v.*
GRAY, J. (1932) *J. exp. Biol.*, **9**, 277–299. *131.*
GREENWOOD, A. W. & BLYTH, J. S. S. (1951) *Heredity*, **5**, 215–231. *105.*
GUSTAFSON, T. & HASSELBERG, I. (1950) *Exp. Cell Res.*, **1**, 371–375. *86.*
GUSTAFSON, T. & HJELTE, M.-B. (1951) *Exp. Cell Res.*, **2**, 474–490. *87.*
HAGSTRÖM, B. & ALLEN, R. D. (1956) *Exp. Cell Res.*, to be published. *14, 117.*
HAGSTRÖM, B. & HAGSTRÖM, B. (1954*a*) *Exp. Cell Res.*, **6**, 479–484. *111.*
HAGSTRÖM, B. & HAGSTRÖM, B. (1954*b*) *Exp. Cell Res.*, **6**, 491–496. *121.*
HAGSTRÖM, B. & HAGSTRÖM, B. (1954*c*) *Exp. Cell Res.*, **6**, 532–534. *14, 137.*
HAMILTON, H. H. (1952) *Lillie's Development of the Chick.* Henry Holt & Co., New York. *103.*
HAMILTON, W J. & DAY, F. T. (1945) *J. Anat., London*, **79**, 127–130. *3.*
HARDING, C. V. & HARDING, D. (1952*a*) *Ark. Zool.*, **4**, No. 3, 91–93. *137.*
HARDING, C. V. & HARDING, D. (1952*b*) *Exp. Cell Res.*, **3**, 475–484. *137.*
HARDING, D. (1949) *Proc. Soc. exp. Biol., N.Y.*, **71**, 14–15. *96.*
HARDING. D. (1951) *Nature, Lond.*, **167**, 355. *96.*
HARTMANN, M. (1944) *Naturwissenschaften*, **32**, 231. *23.*
HARTMANN, M., MEDEM, F. Graf von, KUHN, R. & BIELIG, H.-J. (1947) *Naturwissenschaften*, **1**, 25. *33.*
HARTMANN, M. & SCHARTAU, O. (1939) *Biol. Zbl.*, **59**, 571–587. *36.*
HARTMANN, M., SCHARTAU, O. & WALLENFELS, K. (1940) *Biol. Zbl.*, **60**, 398–423. *33, 38.*
HARTREE, E. F. (1953) *J. Amer. chem. Soc.*, **75**, 6244–6249. *49.*
HARVEY, E. B. (1938) *Biol. Bull., Wood's Hole*, **75**, 170–188. *8.*
HARVEY, E. B. (1941) *Biol. Bull., Wood's Hole*, **80**, 354–362. *83.*
HARVEY, E. N. (1910) *J. exp. Zool.*, **8**, 355–376. *98.*
HARVEY, E. N. (1911) *J. exp. Zool.*, **10**, 507–556. *10.*
HARVEY, E. N. (1931) *Biol. Bull., Wood's Hole*, **61**, 273–279. *vi, 93.*
HASAMA, B. (1935) *Protoplasma*, **22**, 597–606. *128.*
HAYASHI, T. (1946) *Biol. Bull., Wood's Hole*, **90**, 177–187. *33.*
HAYES, F. R. (1938) *Biol. Bull., Wood's Hole*, **74**, 267–277. *87.*
HAYES, F. R., DARCY, D. A. & SULLIVAN, C. M. (1946) *J. biol. Chem.*, **163**, 621–631. *84.*
HEILBRUNN, L. V. (1915) *Biol. Bull., Wood's Hole*, **29**, 149–203. *94.*
HEILBRUNN, L. V. (1921) *J. exp. Zool.*, **34**, 417–447. *95.*
HEILBRUNN, L. V. (1934) *Biol. Bull., Wood's Hole*, **66**, 264–275. *98.*
HEILBRUNN, L. V. (1952) *An Outline of General Physiology.* W. B. Saunders Philadelphia. *v, 89, 94, 95, 124.*
HEILBRUNN, L. V., MAZIA, D. & STEINBACH, H. B. (1934) *Anat. Rec.*, **60**, Abstracts, p. 32. *83.*
HEILBRUNN, L. V. & WILBUR, K. M. (1937) *Biol. Bull., Wood's Hole*, **73**, 557–564. *98.*
HEILBRUNN, L. V. & WILSON, W. L. (1948) *Biol. Bull., Wood's Hole*, **95**, 57–68. *95, 96.*
HENLE, W., HENLE, G. & CHAMBERS, L. A. (1938) *J. exp. Med.*, **68**, 335–352. *23.*

HERLANT, M. (1920) *Arch. Biol., Paris*, **30**, 517–600. *99–102.*
HERTWIG, O. & HERTWIG, R. (1887) *Jena Z. Naturw.*, **20**, 120–242. *116.*
HIBBARD, H. (1928) *Arch. Biol., Paris*, **38**, 251–326. *36.*
HIRAMOTO, Y. (1954) *Jap. J. Zool.*, **2**, 227–243. *11.*
HIRST, G. K. (1942) *J. exp. Med.*, **75**, 49–64. *28.*
HISHIDA, T. & NAKANO, E. (1954) *Embryologia*, **2**, 67–79. *78, 84.*
HÖBER, R. (1945) *Physical Chemistry of Cells and Tissues*. J. & A. Churchill Ltd., London. *v.*
HOBSON, A. D. (1928) *Brit. J. exp. Biol.*, **6**, 65–78. *20, 98.*
HOBSON, A. D. (1932*a*) *J. exp. Biol.*, **9**, 69–92. *80, 100, 101.*
HOBSON, A. D. (1932*b*) *J. exp. Biol.*, **9**, 93–106. *98.*
HODGKIN, A. L. & HUXLEY, A. F. (1945) *J. Physiol.*, **104**, 176–195. *125.*
HODGKIN, A. L. & HUXLEY, A. F. (1952) *J. Physiol.*, **117**, 500–544. *127.*
HOFF-JØRGENSEN, E. (1954) *Recent Developments in Physiology*. Butterworths Scientific Publications, London. *85.*
HOLLANDER, W. F. (1949) *J. Hered.*, **40**, 271–278. *105.*
HOLTER, H. (1936) *J. cell. comp. Physiol.*, **8**, 179–200. *87.*
HOLTER, H. & ZEUTHEN, E. (1944) *C.R. Lab. Carlsberg*, **25**, 33–65. *57, 61.*
HOLZER, W. (1933) *Pflüg. Arch. ges. Physiol.*, **232**, 835–841. *131.*
HORECKER, B. L. (1953) *Brew. Dig.*, **28**, 214–219. *72.*
HOROWITZ, N. H. (1940) *J. cell. comp. Physiol.*, **15**, 299–308. *65.*
HOROWITZ, N. H. & BAUMBERGER, J. P. (1941) *J. biol. Chem.*, **141**, 407–415. *67.*
HÖRSTADIUS, S. (1923) *Arch. EntwMech. Org.*, **98**, 1–9. *98.*
HÖRSTADIUS, S. (1939) *Pubbl. Staz. zool. Napoli*, **17**, 221–312. *7.*
HÖRSTADIUS, S. & RUNNSTRÖM, J. (1953) *Exp. Cell Res.*, **4**, 468–476. *118.*
HULTIN, E., KRISZAT, G., LINDVALL, S., LUNDBLAD, G., LÖW, H., RUNNSTRÖM, J., VASSEUR, E. & WICKLUND, E. (1952) *Ark. Kemi.*, **5**, 83–87. *30.*
HULTIN, T. (1947) *Pubbl. Staz. zool. Napoli*, **21**, 153–163. *34.*
HULTIN, T. (1948) *Ark. Zool.*, **40A**, No. 20. *137, 138.*
HULTIN, T. (1949) *Ark. Kemi. Min. Geol.*, **26A**, No. 27. *98.*
HULTIN, T. (1950*a*) *Exp. Cell Res.*, **1**, 159–168. *98.*
HULTIN, T. (1950*b*) *Exp. Cell Res.*, **1**, 272–283. *98.*
HULTIN, T. (1950*c*) *Exp. Cell Res.*, **1**, 599–602. *76.*
HULTIN, T. (1953*a*) *Studies on the Structure and Metabolic Background of Fertilization and Development*. Stockholm. *73, 76.*
HULTIN, T. (1953*b*) *Ark. Kemi.*, **6**, No. 15, 195–200. *79.*
HUTCHENS, J. O., KELTCH, A. K., KRAHL, M. E. & CLOWES, G. H. A. (1942) *J. gen. Physiol.*, **25**, 717–731. *70, 86, 87.*
IIDA, T. T. (1943*a*) *J. Fac. Sci. Tokyo Univ., Sectn. IV Zoology*, **6**, 141–151. *131, 132.*
IIDA, T. T. (1943*b*) *J. Fac. Sci. Tokyo Univ., Sectn. IV Zoology*, **6**, 153–163. *131.*
IIDA, T. T. (1943*c*) *J. Fac. Sci. Tokyo Univ., Sectn. IV Zoology*, **6**, 165–173. *133, 134.*
IMMERS, J. (1949) *Ark. Zool.*, **42A**, No. 6. *97.*
IMMERS, J. & VASSEUR, E. (1949) *Experientia*, **5**, 124–125. *25.*
INFANTELLINA, F. & LA GRUTTA, G. (1948) *Arch. Sci. biol.*, **32**, 85–106. *77, 90.*
ISHIDA, J. (1954) *J. Fac. Sci. Tokyo Univ., Sectn. IV Zoology*, **7**, 53–59. *37.*
ISHIKAWA, M. (1954) *Embryologia*, **2**, 57–62. *80, 81.*
JANDORF, B. J. & KRAHL, M. E. (1942) *J. gen. Physiol.*, **25**, 749–754. *70, 73, 84.*
JORDAN, E. O. (1893) *J. Morph.*, **8**, 269–366. *103.*
JUST, E. E. (1914) *Biol. Bull., Wood's Hole*, **27**, 201–212. *2.*
JUST, E. E. (1919) *Biol. Bull., Wood's Hole*, **36**, 1–10. *9.*
JUST, E. E. (1928) *Physiol. Zool.*, **1**, 26–36. *101.*

JUST, E. E. (1929) *Biol. Bull., Wood's Hole*, **57**, 311–325. *7*.
JUST, E. E. (1939) *The Biology of the Cell Surface*. The Technical Press Ltd., London. *38*.
KACSER, H. (1955) *J. exp. Biol.*, **32**, 451–467. *8*, *108*, *119*.
KAVANAU, J. L. (1953) *J. exp. Zool.*, **122**, 285–337. *76–78*.
KAVANAU, J. L. (1954*a*) *Exp. Cell Res.*, **6**, 563–566. *76–78*.
KAVANAU, J. L. (1954*b*) *Exp. Cell Res.*, **7**, 530–557. *62*, *76–78*.
KEILIN, D. & HARTREE, E. F. (1939) *Proc. roy. Soc. B*, **127**, 167–191. *67*.
KELTCH, A. K., STRITTMATTER, C. F., WALTERS, C. P. & CLOWES, G. H. A. (1950) *J. gen. Physiol.*, **33**, 547–553. *78*.
KRAHL, M. E., JANDORF, B. J. & CLOWES, G. H. A. (1942) *J. gen. Physiol.*, **25**, 733–747. *78*, *84*.
KRAHL, M. E., KELTCH, A. K., NEUBECK, C. E. & CLOWES, G. H. A. (1941) *J. gen. Physiol.*, **24**, 597–617. *67*.
KRAHL, M. E., KELTCH, A. K., WALTERS, C. P. & CLOWES, G. H. A. (1953) *Biol. Bull., Wood's Hole*, **105**, 377. *70*.
KRAHL, M. E., KELTCH, A. K., WALTERS, C. P. & CLOWES, G. H. A. (1954*a*) *Biol. Bull., Wood's Hole*, **107**, 315–316. *70*.
KRAHL, M. E., KELTCH, A. K., WALTERS, C. P. & CLOWES, G. H. A. (1954*b*) *J. gen. Physiol.*, **38**, 31–39. *70*, *89*.
KRAHL, M. E., KELTCH, A. K., WALTERS, C. P. & CLOWES, G. H. A. (1955) *J. gen. Physiol.*, **38**, 431–439. *71*.
KRAUSS, M. (1950) *Science*, **112**, 759. *37*.
KRUGELIS, E. J. (1950) *C.R. Lab. Carlsberg*, **27**, 273–290. *86*.
KUHN, R. & LÖW, I. (1949*a*) *Chem. Ber.*, **82**, 474–479. *51*.
KUHN, R. & LÖW, I. (1949*b*) *Chem. Ber.*, **82**, 479–481. *51*.
KUPELWIESER, H. (1909) *Arch. EntwMech. Org.*, **27**, 434–462. *139*.
KUSA, M. (1953) *Annot. zool. jap.*, **26**, 73–77. *6*, *80*.
KUSA, M. (1954) *Annot. zool. jap.*, **27**, 1–6. *6*.
KUSHNER, KH. (1954) *Izvestiya Akademii Nauk SSSR* (*Ser. biol.*), No. 1, 32–52. *122*, *123*.
LASER, H. & ROTHSCHILD, Lord (1939) *Proc. roy. Soc. B*, **126**, 539–557. *63–65*.
LASLEY, J. F., EASLEY, G. T. & MCKENZIE, F. F. (1942) *Anat. Rec.*, **82**, 167–174. *109*.
LEWIS, D. (1954) *Advanc. Genet.*, **6**, 235–285. *53*.
LILLIE, F. R. (1912*a*) *Science*, **36**, 527–530. *21*.
LILLIE, F. R. (1912*b*) *J. exp. Zool.*, **12**, 413–478. *1*, *12*.
LILLIE, F. R. (1914) *J. exp. Zool.*, **16**, 523–590. *34*.
LILLIE, F. R. (1919) *Problems of Fertilization*. University of Chicago Press, Chicago. *6*, *23*, *31*, *38*, *139*, *140*.
LILLIE, R. S. (1916) *Amer. J. Physiol.*, **40**, 249–266. *80*.
LILLIE, R. S. (1924) *General Cytology* (ed. E. V. Cowdry). University of Chicago Press, Chicago. *124*.
LINDAHL, P. E. (1938) *Naturwissenschaften*, **26**, 709–710. *68*.
LINDAHL, P. E. (1940) *Ark. Kemi. Min. Geol.*, **14A**, No. 12. *67*.
LINDAHL, P. E. & RUNNSTROM, J. (1929) *Acta zool., Stockh.*, **10**, 401–484. *158*.
LINDBERG, O. (1943) *Ark. Kemi. Min. Geol.*, **16A**, No. 15. *87*.
LINDBERG, O. (1945) *Ark. Kemi. Min. Geol.*, **20B**, No. 1. *86*, *87*.
LINDBERG, O. (1948) *Ark. Kemi. Min. Geol.*, **26B**, No. 13. *82*.
LINDBERG, O. & ERNSTER, L. (1948) *Biochim. biophys. Acta*, **2**, 471–477. *69*, *71*, *73*.
LINDERSTRØM-LANG, K. & HOLTER, H. (1933) *Z. physiol. Chem.*, **215**, 167–178. *87*.
LINDVALL, S. & CARSJÖ, A. (1948) *Ark. Kemi. Min. Geol.*, **26B**, No. 12. *87*.

LINDVALL, S. & CARSJÖ, A. (1951) *Exp. Cell Res.*, **2**, 491–498. *83, 89.*
LINDVALL, S. & CARSJÖ, A. (1954) *Ark. Kemi.*, **7**, 17–27. *88.*
LOEB, J. (1903) *Pflüg. Arch. ges. Physiol.*, **99**, 323–356. *136.*
LOEB, J. (1913) *Artificial Parthenogenesis and Fertilization.* University of Chicago Press, Chicago. *1.*
LOEB, J. (1914) *Science*, **40**, 316–318. *98.*
LOEB, J. & WASTENEYS, H. (1913) *Arch. EntwMech. Org.*, **35**, 555–557. *56.*
LOVELACE, R. (1949) *Biol. Bull., Wood's Hole*, **97**, 259. *85.*
LUCKÉ, B., HARTLINE, H. K. & MCCUTCHEON, M. (1931) *J. gen. Physiol.*, **14**, 405–419. *80.*
LUCKÉ, B. & MCCUTCHEON, M. (1932) *Physiol. Rev.*, **12**, 68–139. *80.*
LUNDBERG, A. (1956) *Exp. Cell Res.*, to be published. *130, 131.*
LUNDBLAD, G. (1949) *Nature, Lond.*, **163**, 643. *87–88.*
LUNDBLAD, G. (1950) *Exp. Cell Res.*, **1**, 264–271. *87–88.*
LUNDBLAD, G. (1954*a*) *Ark. Kemi.*, **7**, 127–157. *87–88.*
LUNDBLAD, G. (1954*b*) *Ark. Kemi.*, **7**, 159–167. *87–88.*
LUNDBLAD, G. (1954*c*) *Ark. Kemi.*, **7**, 169–180. *87–88.*
LUNDBLAD, G. (1954*d*) *Proteolytic Activity in Sea-Urchin Gametes.* Uppsala. *87–88.*
LUNDBLAD, G. & HULTIN, E. (1954) *Exp. Cell Res.*, **6**, 249–250. *88.*
LUNDBLAD, G. & LUNDBLAD, I. (1953) *Ark. Kemi.*, **6**, 387–415. *87–88.*
MANERY, J. F. & IRVING, L. (1935) *J. cell. comp. Physiol.*, **5**, 457–464. *84.*
MANN, T. (1951) *Biochem. Soc. Symposia*, No. **7**, 11–21. *69.*
MANN, T. (1954) *Biochemistry of Semen.* Methuen, London. *33.*
MARSHAK, A. & FAGER, J. (1950) *J. cell. comp. Physiol.*, **35**, 317–329. *85.*
MARSLAND, D. A. (1939) *J. cell. comp. Physiol.*, **13**, 15–22. *101.*
MARTIN, G. J. & BEILER, J. M. (1952) *Science*, **115**, 402. *54.*
MATHEWS, A. P. (1913) *J. biol. Chem.*, **14**, 465–467. *75.*
MAZIA, D. (1937) *J. cell. comp. Physiol.*, **10**, 291–304. *83.*
MAZIA, D., BLUMENTHAL, G. & BENSON, E. (1948) *Biol. Bull., Wood's Hole*, **95**, 250–251. *86.*
MEDEM, F. Graf von (1942) *Biol. Zbl.*, **62**, 431–446. *23.*
MEDEM, F. Graf von, RÖTHELI, A. & ROTH, H. (1949) *Schweiz. Z. Hydrol.*, **11**, 361–377. *23.*
METZ, C. B. (1945) *Biol. Bull., Wood's Hole*, **89**, 84–94. *29.*
METZ, C. B. (1949) *Proc. Soc. exp. Biol., N.Y.*, **70**, 422–424. *34.*
METZ, C. B. & DONOVAN, J. (1951) *Biol. Bull., Wood's Hole*, **101**, 202. *30.*
MEVES, F. (1912) *Arch. mikr. Anat.*, **80**, 81–123. *12.*
MINGANTI, A. (1951) *Pubbl. Staz. zool. Napoli*, **23**, 58–65. *23.*
MIRSKY, A. E. (1936) *Science*, **84**, 333–334. *88.*
MITCHISON, J. M. (1953) *Exp. Cell Res.*, **5**, 536–538. *9.*
MITCHISON, J. M. (1956) *Quart. J. micr. Sci.*, to be published. *92.*
MITCHISON, J. M. & SWANN, M. M. (1952) *J. exp. Biol.*, **29**, 357–362. *91, 101, 102.*
MITCHISON, J. M. & SWANN, M. M. (1953) *Quart. J. micr. Sci.*, **94**, 381–389. *11, 90.*
MITCHISON, J. M. & SWANN, M. M. (1954*a*) *J. exp. Biol.*, **31**, 443–460. *92, 93, 100, 133.*
MITCHISON, J. M. & SWANN, M. M. (1954*b*) *J. exp. Biol.*, **31**, 461–472. *vi, 92, 93.*
MITCHISON, J. M. & SWANN, M. M. (1955) *J. exp. Biol.*, **32**, 734–750. *92–95, 101.*
MOEWUS, F. (1949) *Portug. acta biol., Ser. A* (R. B. Goldschmidt Volume), 161–199. *51.*

Moewus, F. (1950) *Biol. Zbl.*, **69**, 181–197. *51, 54.*
Monné, L. & Hårde, S. (1951) *Ark. Zool.*, **1**, 487–498. *6.*
Monroy, A. (1945) *Experientia*, **1**, 335–336. *101.*
Monroy, A. (1948) *Ark. Zool.*, **40A**, No. 21. *8, 37.*
Monroy, A. (1950) *Exp. Cell Res.*, **1**, 92–104. *89.*
Monroy, A. (1954) *Pubbl. Staz. zool. Napoli*, **25**, 188–197. *20.*
Monroy, A. & Monroy-Oddo, A. (1951) *J. gen. Physiol.*, **35**, 245–253. *89.*
Monroy, A. & Montalenti, G. (1947) *Biol. Bull., Wood's Hole*, **92**, 151–161. *100, 101.*
Monroy, A. & Ruffo, A. (1945) *Boll. Soc. ital. Biol. sper.*, **20**, 406–407. *75, 87.*
Monroy, A. & Ruffo, A. (1947) *Nature, Lond.*, **159**, 603. *36, 37.*
Monroy, A. & Runnström, J. (1950) *Biol. Bull., Wood's Hole*, **99**, 339. *35.*
Monroy, A. & Tosi, L. (1952) *Experientia*, **8**, 393–394. *37.*
Monroy, A., Tosi, L., Giardina, G. & Maggio, R. (1954) *Biol. Bull., Wood's Hole*, **106**, 169–177. *26, 30, 31.*
Monroy-Oddo, A. (1946) *Experientia*, **2**, 371–372. *83.*
Monroy-Oddo, A. & Esposito, M. (1951) *J. gen. Physiol.*, **34**, 285–293. *83.*
Moore, A. R. (1937) *Protoplasma*, **27**, 544–551. *18.*
Morgan, L. V. (1929) *Carnegie Inst. Washington Pub.*, No. 399, 223–296. *105.*
Morgan, T. H. (1927) *Experimental Embryology*. Columbia University Press, New York. *105.*
Morgan, T. H. & Tyler, A. (1930) *Biol. Bull., Wood's Hole*, **58**, 59–73. *19.*
Moser, F. (1939*a*) *J. exp. Zool.*, **80**, 423–445. *6, 107.*
Moser, F. (1939*b*) *J. exp. Zool.*, **80**, 447–471. *98.*
Motomura, I. (1936) *Zool. Mag., Tokyo*, **48**, 753–758. *9.*
Motomura, I. (1941) *Sci. Rep. Tôhoku Univ., 4th Ser., B*, **16**, 345–363. *9.*
Motomura, I. (1950) *Sci. Rep. Tôhoku Univ., 4th Ser., B*, **18**, 561–570. *10, 24.*
Motomura, I. (1953*a*) *Sci. Rep. Tôhoku Univ., 4th Ser., B*, **20**, 93–97. *24.*
Motomura, I. (1953*b*) *Exp. Cell Res.*, **5**, 187–190. *24.*
Motomura, I. (1954) *Sci. Rep. Tôhoku Univ., 4th Ser., B*, **20**, 158–162. *10.*
Des Moulins, C. (1835–37) *Act. Soc. linn. Bordeaux*, **3**, 126. *154.*
Mudd, E. B. H., Mudd, S. & Keltch, A. H. (1929) *Proc. Soc. exp. Biol., N.Y.*, **26**, 392–394. *30.*
Nakano, E. (1953) *Embryologia*, **2**, 21–31. *62.*
Nakano, E. (1954) *Jap. J. Zool.*, **11**, 245–251. *113.*
Nakano, E. & Ohashi, S. (1954) *Embryologia*, **2**, 81–86. *26.*
Needham, J. (1931) *Chemical Embryology*, University Press, Cambridge. *100.*
Needham, J. (1942) *Biochemistry and Morphogenesis*, University Press, Cambridge. *58, 65, 68.*
Nelson, T. C. (1941) *Anat. Rec.*, **81**, Abstracts, p. 88. *21.*
Nelson, T. C. & Allison, J. B. (1937) *Anat. Rec.*, **70**, Abstract p. 124. *21.*
Novikoff, A. B. (1939) *J. exp. Zool.*, **82**, 217–237. *6.*
Numanoi, H. (1953*a*) *Sci. Papers Coll. Gen. Ed. Univ. Tokyo*, **3**, 55–65. *89.*
Numanoi, H. (1953*b*) *Sci. Papers Coll. Gen. Ed. Univ. Tokyo*, **3**, 193–200. *89.*
Öhman, L. O. (1940) *Ark. Zool.*, **32A**, No. 15. *65.*
Ohman, L. O. (1942) *Naturwissenschaften*, **30**, 240. *74.*
Öhman, L. O. (1945) *Ark. Zool.*, **36A**, No. 7. *74, 75, 86, 87, 101.*
Okkelberg, P. (1914) *Biol. Bull., Wood's Hole*, **26**, 92–99. *5.*
Oppel, A. (1892) *Arch. mikr. Anat.*, **39**, 215–290. *103.*
Örström, Å. (1941) *Z. physiol. Chem.*, **271**, 1–176. *75, 76, 86.*
Örström, Å. & Lindberg, O. (1940) *Enzymologia*, **8**, 367–384. *70, 73, 74, 82, 84, 87.*
Örström, Å. & Örström, M. (1942) *Protoplasma*, **36**, 475–490. *83.*
Osterhout, W. J. V. (1950) *Biol. Bull., Wood's Hole*, **99**, 362. *36.*

OSTERHOUT, W. J. V. (1952) *Biol. Bull., Wood's Hole*, **103**, 305–306. *36.*
OSTERHOUT, W. J. V. (1953) *Biol. Bull., Wood's Hole*, **105**, 379–380. *36.*
PAGE, I. H. (1923) *J. biol. Chem.*, **57**, 471–476. *75.*
PAGE, I. H. (1929) *Brit. J. exp. Biol.*, **6**, 219–228. *101.*
PANIJEL, J. (1947) *Bull. Soc. Chim. biol., Paris*, **29**, 1098–1106. *85.*
PANIJEL, J. (1951) *Métabolisme des Nucléoprotéines.* Hermann & Cie, Paris. *87.*
PARAT, M. (1933) *C.R. Soc. Biol., Paris*, **112**, 1134–1137. *36.*
PARPART, A. K. (1941) *Biol. Bull., Wood's Hole*, **81**, 296. *87.*
PASTEELS, J. J. (1935) *Arch. Biol., Paris*, **46**, 229–262. *98.*
PASTEELS, J. (1938) *Trav. Sta. zool. Wimereux*, **13**, 515–530. *98.*
PASTEELS, J. (1948) *Arch. Biol., Paris*, **59**, 405–446. *85.*
PASTEELS, J. (1950) *Arch. Biol., Paris*, **61**, 197–220. *10.*
PASTEELS, J. & LISON, L. (1951) *Nature, Lond.*, **167**, 948–949. *85.*
PAULING, L., CAMPBELL, D. H. & PRESSMAN, D. (1943) *Physiol. Rev.*, **23**, 203–219. *137.*
PEACOCK, A. D. (1944) *Proc. R. phil. Soc. Glasg.*, **68**, 99–124. *1.*
PEARSON, O. P. & ENDERS, R. K. (1943) *Anat. Rec.*, **85**, 69–83. *2.*
PERLZWEIG, W. A. & BARRON, E. S. G. (1928) *J. biol. Chem.*, **79**, 19–26. *70.*
PÉTERFI, T. & ROTHSCHILD, V. (1935) *Nature, Lond.*, **135**, 874–875. *128.*
PFEFFER, W. (1884) *Unt. Bot. Inst. Tübingen*, **1**, 363–481. *39, 47.*
PHILIPS, F. S. (1940) *Biol. Bull., Wood's Hole*, **78**, 256–274. *61.*
PICKEN, L. E. R. & ROTHSCHILD, Lord (1948) *J. exp. Biol.*, **25**, 227–236. *81.*
PINCUS, G. (1936) *The Eggs of Mammals.* The Macmillan Co., New York. *20.*
POPA, G. T. (1927) *Biol. Bull., Wood's Hole*, **52**, 238–257. *30.*
QUAYLE, D. B. (1940) *Progr. Rep. biol. Stas. Nanaimo and Prince Rupert*, **43**, 8–9. *21.*
RACE, R. R. (1944) *Nature, Lond.*, **153**, 771–772. *29.*
RICHARDS, A. G. & MILLER, A. (1937) *J.N.Y. ent. Soc.*, **45**, 1–60. *103.*
ROBBIE, W. A. (1946) *J. cell. comp. Physiol.*, **28**, 305–324. *67.*
ROTH, J. S. (1953) *Nature, Lond.*, **171**, 127–128. *88.*
ROTHSCHILD, Lord (1938) *J. exp. Biol.*, **15**, 209–216. *129.*
ROTHSCHILD, Lord (1939) *J. exp. Biol.*, **16**, 49–55. *66.*
ROTHSCHILD, Lord (1946) *J. exp. Biol.*, **23**, 77–99. *131, 135.*
ROTHSCHILD, Lord (1948) *J. exp. Biol.*, **25**, 344–352. *37, 38.*
ROTHSCHILD, Lord (1949*a*) *J. exp. Biol.*, **26**, 100–111. *66, 67.*
ROTHSCHILD, Lord (1949*b*) *J. exp. Biol.*, **26**, 177–181. *118.*
ROTHSCHILD, Lord (1950) *J. exp. Biol.*, **26**, 410–411. *90.*
ROTHSCHILD, Lord (1951) *Biol. Rev.*, **26**, 1–27. *34, 38, 41.*
ROTHSCHILD, Lord (1952) *Int. Rev. Cytology*, **1**, 257–263. *33, 40–43.*
ROTHSCHILD, Lord (1953) *J. exp. Biol.*, **30**, 57–67. *1, 18, 117.*
ROTHSCHILD, Lord & BARNES, H. (1953) *J. exp. Biol.*, **30**, 534–544. *129.*
ROTHSCHILD, Lord & CLELAND, K. W. (1952) *J. exp. Biol.*, **29**, 66–67. *12.*
ROTHSCHILD, Lord & SWANN, M. M. (1949) *J. exp. Biol.*, **26**, 164–176. *6, 107, 108, 117.*
ROTHSCHILD, Lord & SWANN, M. M. (1951) *J. exp. Biol.*, **28**, 403–416. *30, 110, 111.*
ROTHSCHILD, Lord & SWANN, M. M. (1952) *J. exp. Biol.*, **29**, 469–483. *112, 120.*
ROTHSCHILD, Lord & TYLER, A. (1954) *J. exp. Biol.*, **31**, 252–259. *31.*
ROTHSCHILD, Lord & TYLER, A. (1955) *Exp. Cell Res.*, Suppl., **3**, 304–311. *8.*
ROWEN, J. W. & KORNBERG, A. (1951) *J. biol. Chem.*, **193**, 497–507. *73.*
RÜCKERT, J. (1899) *Festschr. zum 70 Geburtstag von Carl von Kuppfer, Jena*, 581–704. *103.*
RUDENBERG, F. H. N. (1953) *Exp. Cell Res.*, **4**, 116–126. *83.*
RUNNSTRÖM, J. (1924) *Acta. zool., Stockh.*, **5**, 345–392. *101.*

RUNNSTRÖM, J. (1928) *Protoplasma*, **4**, 388–514. *91, 107.*
RUNNSTRÖM, J. (1930) *Protoplasma*, **10**, 106–173. *58, 67.*
RUNNSTRÖM, J. (1933) *Biochem. Z.*, **258**, 257–279. *65, 70, 74, 82, 84.*
RUNNSTRÖM, J. (1935*a*) *Biol. Bull., Wood's Hole*, **68**, 327–334. *58, 85.*
RUNNSTRÖM, J. (1935*b*) *Biol. Bull., Wood's Hole*, **69**, 345–350. *34.*
RUNNSTRÖM, J. (1949) *Advanc. Enzymol.*, **9**, 241–327. *vii, 35, 74.*
RUNNSTRÖM, J. (1951) *Harvey Lectures, Series 46*, 116–152. *10.*
RUNNSTRÖM, J. (1952) *Symp. Soc. exp. Biol.*, **6**, 39–88. *10, 35.*
RUNNSTRÖM, J. & KRISZAT, G. (1950) *Exp. Cell Res.*, **1**, 355–370. *97.*
RUNNSTRÖM, J. & KRISZAT, G. (1952) *Exp. Cell Res.*, **3**, 419–426. *118.*
RUNNSTRÖM, J., LINDVALL, S. & TISELIUS, A. (1944*a*) *Nature, Lond.*, **153**, 285. *38.*
RUNNSTRÖM, J., MONNÉ, L. & BROMAN, L. (1944*b*) *Ark. Zool.*, **35A**, No. 3. *91, 93, 101, 137.*
RUNNSTRÖM, J., MONNÉ, L. & WICKLUND, E. (1946) *J. Colloid Sci.*, **1**, 421–452. *91.*
RUNNSTRÖM, J., TISELIUS, A. & LINDVALL, S. (1945) *Ark. Zool.*, **36A**, No. 22. *36.*
RUNNSTRÖM, J., TISELIUS, A. & VASSEUR, E. (1942) *Ark. Kemi. Min. Geol.*, **15A**, No. 16. *24, 35.*
RYBAK, B. (1949) *Bull. Soc. Chim. biol., Paris*, **31**, 464–484. *30.*
SAMPSON, M. M. (1922) *Biol. Bull., Wood's Hole*, **43**, 267–284. *29.*
SAMPSON, M. M. (1926*a*) *Biol. Bull., Wood's Hole*, **50**, 202–206. *120.*
SAMPSON, M. M. (1926*b*) *Biol. Bull., Wood's Hole*, **50**, 301–338. *120.*
SCHARTAU, O. & MONTALENTI, G. (1941) *Biol. Zbl.*, **61**, 473–478. *23.*
SCHEER, B. T. (1945) *Science*, **101**, 645–646. *89.*
SCHEER, B. T., MONROY, A., SANTANGELO, M. & RICCOBONO, G. (1954) *Exp. Cell Res.*, **7**, 284–287. *128, 130.*
SCHEER, B. T. & SCHEER, M. A. R. (1947) *Physiol. Zool.*, **20**, 15–32. *89.*
SCHMIDT, G., HECHT, L. & THANNHAUSER, S. J. (1948) *J. gen. Physiol.*, **31**, 203–207. *84.*
SEKINE, T. (1951) *J. Biochem., Tokyo*, **38**, 171–179. *89.*
SEXTON, W. A. (1949) *Chemical Constitution and Biological Activity*. E. & F. N. Spon, Ltd., London. *86.*
SHAPIRO, H. (1939) *Biol. Bull., Wood's Hole*, **77**, 317–318. *81.*
SHAPIRO, H. (1948) *J. gen. Physiol.*, **32**, 43–51. *80.*
SHAVER, J. R. (1949) *J. Cyto-embryol. belgo-néerland*, pp. 61–66. *96.*
SHETTLES, L. B. (1954) *Trans. New York Acad. Sci.*, **17**, 99–102. *12, 13.*
SHIBATA, K. (1911) *Jb. wiss. Bot.*, **49**, 1. *46.*
SIEVE, B. F. (1952) *Science*, **116**, 373–385. *54.*
SMITH, A. U. (1951) *Biochem. Soc. Symposia*, **7**, 3–10. *13.*
SMITHBERG, M. (1953) *Anat. Rec.*, **117**, Abstracts p. 554. *12.*
SOUTHWICK, W. E. (1939) *Biol. Bull., Wood's Hole*, **77**, 147–156. *37.*
SPIEGELMAN, S. & STEINBACH, H. B. (1945) *Biol. Bull., Wood's Hole*, **88**, 254–268. *78.*
SPIKES, J. D. (1949*a*) *Biol. Bull., Wood's Hole*, **97**, 95–99. *29.*
SPIKES, J. D. (1949*b*) *Amer. Nat.*, **83**, 285–301. *33.*
STEFANELLI, A. (1938) *Arch. Sci. biol.*, **24**, 411–441. *61.*
STEINERT, M. (1952) *Bull. Soc. Chim. biol., Paris*, **34**, 923–936. *86.*
STRICHT, O. van der (1902) *Verh. anat. Ges. Jena*, **21**, 163–168. *12.*
STRICHT, O. van der (1923) *Arch. Biol., Paris*, **33**, 229–300. *2.*
SUGIYAMA, M. (1951) *Biol. Bull., Wood's Hole*, **101**, 335–344. *119, 120.*
SUGIYAMA, M. (1953) *Biol. Bull., Wood's Hole*, **104**, 210–215. *80.*
SWANN, M. M. (1952) *Symp. Soc. exp. Biol.*, **6**, 89–104. *101.*

SWIFT, H. (1953) *Int. Rev. Cytology*, **2**, 1–76. *85.*
TUZET, O. (1950) *Vie et Milieu*, **1**, 163–177. *v, 123.*
TYLER, A. (1932) *J. exp. Zool.*, **63**, 155–173. *19.* Pl. II.
TYLER, A. (1939) *Proc. nat. Acad. Sci., Wash.*, **25**, 317–323. *21, 34, 37.*
TYLER, A. (1940*a*) *Proc. nat. Acad. Sci., Wash.*, **26**, 249–256. *21, 29, 34.*
TYLER, A. (1940*b*) *Biol. Bull., Wood's Hole*, **78**, 159–178. *23, 26.* Pl. III.
TYLER, A. (1941*a*) *Biol. Bull., Wood's Hole*, **81**, 190–204. *28–30.*
TYLER, A. (1941*b*) *Biol. Rev.*, **16**, 291–336. *v.*
TYLER, A. (1942*a*) *West. J. Surg.*, **50**, 126–138. *23, 28.*
TYLER, A. (1942*b*) *Proc. nat. Acad. Sci., Wash.*, **28**, 391–395. *30.*
TYLER, A. (1945) *J. Immunol.*, **51**, 157–172. *29.*
TYLER, A. (1946) *Biol. Bull., Wood's Hole*, **90**, 213–219. *26.*
TYLER, A. (1948) *Physiol. Rev.*, **28**, 180–219. *26–28, 33, 34.*
TYLER, A. (1949) *Amer. Nat.*, **83**, 195–219. *24–26, 35, 138.*
TYLER, A. (1953) *Biol. Bull., Wood's Hole*, **104**, 224–239. *31.*
TYLER, A., BURBANK, A. & TYLER, J. S. (1954) *Biol. Bull., Wood's Hole*, **107**, 303; 304. *25.*
TYLER, A. & FOX, S. W. (1939) *Science*, **90**, 516–517. *23.*
TYLER, A. & FOX, S. W. (1940) *Biol. Bull., Wood's Hole*, **79**, 153–165. *23, 25.*
TYLER, A. & HUMASON, W. D. (1937) *Biol. Bull., Wood's Hole*, **73**, 261–279. *56, 61, 62, 77, 82.*
TYLER, A. & METZ, C. B. (1945) *J. exp. Zool.*, **100**, 387–406. *27.*
TYLER, A. & O'MELVENY, K. (1941) *Biol. Bull., Wood's Hole*, **81**, 364–374. *34, 37.*
TYLER, A., RICCI, N. & HOROWITZ, N. H. (1938) *J. exp. Zool.*, **79**, 129–143. *59, 60.*
TYLER, A. & SCHULTZ, J. (1932) *J. exp. Zool.*, **63**, 509–530. *119.*
ULLYOTT, P. (1936) *J. exp. Biol.*, **13**, 265–278. *40.*
VASSEUR, E. (1948) *Acta chem. scand.*, **2**, 900–913. *26.*
VASSEUR, E. (1950) *Acta chem. scand.*, **4**, 1144–1145. *26.*
VASSEUR, E. (1951) *Acta Borealia*, A. Scientia No. 2, Tromso Museum. *23, 37.*
VASSEUR, E. (1952) Emil Kihlström's Tryckeri A.-B. Stockholm. *24–26, 28, 30, 33.*
VASSEUR, E. & HAGSTRÖM, B. (1946) *Ark. Zool.*, **37A**, No. 17. *37.*
VASSEUR, E. & IMMERS, J. (1949) *Ark. Kemi.*, **1**, 39–41. *26.*
VERNON, H. M. (1900) *Arch. EntwMech. Org.*, **9**, 464–478. *140.*
VILLEE, C. A., LOWENS, M., GORDON, M., LEONARD, E. & RICH, A. (1949) *J. cell. comp. Physiol.*, **33**, 93–112. *84.*
WARBURG, O. (1908) *Zeit. Physiol. Chem.*, **57**, 1–16. *56.*
WARBURG, O. (1911) *Über die Oxydationen in lebenden Zellen nach Versuchen am Seeigelei.* Rössler & Herbert, Heidelberg. *122.*
WHITAKER, D. M. (1931*a*) *J. gen. Physiol.*, **15**, 167–182. *56, 61.*
WHITAKER, D. M. (1931*b*) *J. gen. Physiol.*, **15**, 183–190. *56, 61.*
WHITAKER, D. M. (1933*a*) *J. gen. Physiol.*, **16**, 475–495. *58, 61.*
WHITAKER, D. M. (1933*b*) *J. gen. Physiol.*, **16**, 497–528. *62, 77.*
WHITE, W. E. & CHAMBERS, E. L. (1949) *Rev. path. comp. d'hyg. gen.*, **613**, 647. *70.*
WHITELEY, A. H. (1949) *Amer. Nat.*, **83**, 249–267. *83.*
WICKLUND, E. (1948) *Nature, Lond.*, **161**, 556. *86.*
WICKLUND, E. (1954) *Ark. Zool.*, **7**, 125–129. *31.*
WILKIE, D. (1954) *Exp. Cell Res.*, **6**, 384–391. *47.*
WILSON, E. B. (1902) *Arch. EntwMech. Org.*, **13**, 353–395. *15.*
WILSON, E. B. (1928) *The Cell in Development and Heredity.* The Macmillan Company, New York. *4, 15, 16.*

Wilson, W. L. (1951) *J. cell. comp. Physiol.*, **38**, 409–415. *95*, *101*.
Yamamoto, T. (1944) *Annot. zool. jap.*, **22**, 109–136. *6*.
Yčas, M. (1950) *Studies in the Respiratory Enzymes of Sea-Urchin Eggs*. Ph.D. dissertation, California Institute of Technology. *65*.
Yčas, M. (1954) *J. exp. Biol.*, **31**, 208–217. *67*, *70*, *71*, *78*.
Zeuthen, E. (1944) *C.R. Lab. Carlsberg*, **25**, 191–228. *61*.
Ziegler, H. E. (1898) *Arch. EntwMech. Org.*, **6**, 249–293. *105*.
Zielinski, M. A. (1939) *Acta Biol. exp., Varsovie*, **13**, 35–48. *70*, *73*, *74*, *82*, *87*.

INDEX OF PLANTS AND ANIMALS

The authors of new combinations, and dates, have been omitted; for example, *Limnatis nilotica* (Savigny, 1820) Moquin-Tandon, 1826, would be abbreviated to *Limnatis nilotica* (Savigny). When an author's name is in parentheses after the Latin name of an organism, that author was the first to describe the organism, but under a different name, which has been discarded. Synonyms are restricted to those which occur in gametological papers.

* *Echinus equituberculatus* de Blainville, 1825, p. 86, and *Echinus aequituberculatus* Des Moulins, 1837 p. 126, are synonyms for *Sphaerechinus granularis* (Lamarck). *Echinus aequituberculatus* de Blainville, 1825 p. 76, and *Echinus equituberculatus* de Blainville, 1834 p. 226, are synonyms for *Arbacia lixula* (Linn.).

NAME OF ORGANISM, SYNONYMS AND COMMON NAME, WHEN KNOWN	CLASS, ORDER, ETC.	PAGES
(15) *Balanoglossus kowalevskii* A. Agassiz, see (117)		
(16) *Barnea candida* (Lamarck) WHITE PIDDOCK, ANGEL'S WING	Bivalvia Eulamellibranchiata	98.
(17) *Branchiostoma lanceolatum* (Pallas) *Amphioxus lanceolatus* (Pallas) LANCELET	Cephalochordata	2, 11.
(18) *Brissopsis lyrifera* (Forbes) HEART-URCHIN	Echinoidea Spatangoida	25, 87.
(19) *Bufo bufo* (Linn.) *Bufo vulgaris* Laurenti TOAD	Amphibia Salientia	61.
(20) *Bufo vulgaris* Laurenti, see (19)		
(21) *Cerebratulus lacteus* Verrill RIBBON WORM, PROBOSCIS WORM	Anopla Heteronemertea	5.
(22) *Chaetopterus pergamentaceus* Cuvier, see (23)		
(23) *Chaetopterus variopedatus* (Renier) *Chaetopterus pergamentaceus* Cuvier TUBE-WORM	Polychaeta	10, 20, 56, 58, 61, 81, 95, 96.
(24) *Chlamydomonas eugametos* Moewus	Chlorophyceae Volvocales	52, 54.
(25) *Cimex lectularius* Linn. BED-BUG	Insecta Hemiptera	4.
(26) *Ciona fascicularis* Hancock, see (27)		
(27) *Ciona intestinalis* (Linn.) *Ciona fascicularis* Hancock SEA-SQUIRT	Ascidiacea Enterogona	57, 61, 138.
(28) *Clypeaster japonicus* Döderlein SAND-DOLLAR, CAKE-URCHIN	Echinoidea Clypeastroida	6, 10, 89.
(29) *Crepidula plana* Say FLAT SLIPPER LIMPET, BOAT SHELL	Gastropoda Mesogastropoda	105.
(30) *Cumingia tellinoides* (Conrad) CLAM	Bivalvia Eulamellibranchiata	3, 19, 29, 56, 61, 95.
(31) *Cynthia partita* Stimpson, see (137)		
(32) *Dendraster excentricus* (Eschscholtz) SAND-DOLLAR	Echinoidea Clypeastroida	138, 139.
(33) *Diemictylus palmatus* (Schneider), see (153)		
(34) *Diemictylus viridescens* (Rafinesque) *Molge viridescens* (Rafinesque) *Triturus viridescens* Rafinesque *Triton viridescens* (Rafinesque) NEWT	Amphibia Caudata	103, 104.
(35) *Discoglossus pictus* Otth PAINTED TOAD	Amphibia Salientia	36.
(36) *Dolichoglossus kowalevskii* (A. Agassiz), see (117)		
(37) *Echinarachnius parma* (Lamarck) SAND-DOLLAR	Echinoidea Clypeastroida	26, 87.

NAME OF ORGANISM, SYNONYMS AND COMMON NAME, WHEN KNOWN	CLASS, ORDER, ETC.	PAGES
(63) *Lepidochitona cinerea* (Linn.) GREY MAIL SHELL	Loricata Lepidopleurida	8.
(64) *Lycopodium clavatum* Linn. CLUB-MOSS	Pteridophyta Lycopodiales	50.
(65) *Lymnaea stagnalis* (Linn.) GREAT POND SNAIL	Gastropoda Basommatophora	103.
(66) *Lytechinus pictus* (Verrill) SEA-URCHIN	Echinoidea Diadematoida	24, 29, 35, 71, 82, 83, 138.
(67) *Lytechinus variegatus* (Lamarck) *Toxopneustes variegatus* (Lamarck) SEA-URCHIN	Echinoidea Diadematoida	15.
(68) *Mactra laterialis* Say, see (76)		
(69) *Mactra solidissima* (Dillwyn), see (128)		
(70) *Marthasterias glacialis* (Linn.) *Asterias glacialis* Linn. STARFISH	Asteroidea Forcipulata	61, 98.
(71) *Megathura crenulata* (Sowerby) GIANT (GREAT) KEYHOLE LIMPET	Gastropoda Archaeogastropoda	5, 26, 37.
(72) *Melanopus differentialis* (Thomas) GRASSHOPPER	Insecta Orthoptera	85.
(73) *Mespilia globulus* (Linn.) SEA-URCHIN	Echinoidea Diadematoida	5.
(74) *Molge palmata* (Schneider), see (153)		
(75) *Molge viridescens* (Rafinesque), see (34)		
(76) *Mulinia laterialis* (Say) *Mactra laterialis* Say DWARF SURF CLAM	Bivalvia Eulamellibranchiata	61.
(77) *Mus musculus* Linn. HOUSE MOUSE	Mammalia Rodentia	85.
(78) *Mytilus edulis* (Linn.) EDIBLE MUSSEL	Bivalvia Anisomyaria	3, 8, 37, 139.
(79) *Nereis limbata* Ehlers, see (80)		
(80) *Nereis succinea* (Leuckart) *Nereis limbata* Ehlers	Polychaeta	6, 10, 12, 27, 31, 36, 56, 61, 85, 98.
(81) *Nyctalus noctula* (Schreber) *Vespertilio noctula* Schreber *Vesperugo noctula* (Schreber) COMMON NOCTULE	Mammalia Chiroptera	12.
(82) *Oncorhynchus keta* (Walbaum) DOG SALMON, CHUM	Pisces Isospondyli	6.
(83) *Ornithorhyncus anatinus* (Shaw & Nodder) *Platypus anatinus* Shaw & Nodder *Ornithorhynchus paradoxus* Blumenbach DUCK-BILLED PLATYPUS	Mammalia Monotremata	106.
(84) *Ornithorhynchus paradoxus* Blumenbach, see (83)		

* References are sometimes found to '*Psammechinus miliaris* Z' or '*Psammechinus miliaris* S'. These are private designations in which Z and S refer respectively to littoral and deeper (35 m.) habitats. There are morphological differences between the two forms (Lindahl & Runnström, 1929).

NAME OF ORGANISM, SYNONYMS AND COMMON NAME, WHEN KNOWN	CLASS ORDER, ETC.	PAGES
(105) *Psammechinus pulcherrimus* A. Agassiz, see (56)		
(106) *Psammechinus pustulatus* (L. Agassiz), see (104)		
(107) *Pseudocentrotus depressus* (A. Agassiz) SEA-URCHIN	Echinoidea Diadematoida	26, 89, 131–134.
(108) *Pteridium aquilinum* (Linn.) BRACKEN	Pteridophyta Filicales	39–42, 46, 48–50.
(109) *Rana fusca* Roesel, see (112)		
(110) *Rana pipiens* Schreber LEOPARD FROG	Amphibia Salientia	130, 131.
(111) *Rana platyrrhina* Steenstrup, see (112)		
(112) *Rana temporaria* Linn. *Rana fusca* Roesel *Rana platyrrhina* Steenstrup COMMON FROG	Amphibia Salientia	61, 81, 85–87.
(113) *Rhabditis monohystera* Bütschli	Phasmida Rhabditida	1.
(114) *Sabellaria alveolata* (Linn.) TUBE-WORM	Polychaeta	56, 61, 85, 142.
(115) *Sabellaria vulgaris* Verrill TUBE-WORM	Polychaeta	6.
(116) *Saccoglossus horsti* Brambell & Goodhart ACORN WORM	Enteropneusta	21.
(117) *Saccoglossus kowalewskyi* (A. Agassiz) *Balanoglossus kowalevskii* A. Agassiz *Dolichoglossus kowalevskii* (A. Agassiz) ACORN WORM	Enteropneusta	8, 20.
(118) *Sagitta* Quoy & Gaimard ARROW WORM	Chaetognatha	3.
(119) *Salmo gairdneri* Richardson RAINBOW TROUT	Pisces Isospondyli	84.
(120) *Salmo salar* Linn. SALMON	Pisces Isospondyli	84.
(121) *Salmo trutta* Linn. BROWN TROUT	Pisces Isospondyli	131.
(122) *Salvinia natans* Allioni	Pteridophyta Filicales	46, 48–50.
(123) *Saxostraea commercialis* (Iredale & Roughley) *Ostraea commercialis* Iredale & Roughley ROCK OYSTER	Bivalvia Anisomyaria	56, 61, 64–67, 69.
(124) *Senex interruptus* Ortmann, see (89)		
(125) *Siredon mexicanum* Shaw *Amblystoma mexicanum* (Shaw) AXOLOTL	Amphibia Caudata	86.

* See footnote under (5).

NAME OF ORGANISM, SYNONYMS AND COMMON NAME, WHEN KNOWN	CLASS, UNDER, ETC.	PAGES
(145) *Toxopneustes pictus* Norman, see (129)		
(146) *Toxopneustes variegatus* (Lamarck), see (67)		
(147) *Tripneustes esculentus* (A. Agassiz), see (148)		
(148) *Tripneustes ventricosus* (Lamarck) *Hipponoë esculenta* A. Agassiz *Tripneustes esculentus* (A. Agassiz) SEA-URCHIN	Echinoidea Diadematoida	131, 133.
(149) *Triton alpestris* Laurenti, see (152)		
(150) *Triton palmatus* Schneider, see (153)		
(151) *Triton viridescens* (Rafinesque), see (34)		
(152) *Triturus alpestris* (Laurenti) *Triton alpestris* Laurenti ALPINE NEWT	Amphibia Caudata	1.
(153) *Triturus helveticus* (Razoumowsky) *Diemictylus palmatus* (Schneider) *Molge palmata* (Schneider) *Triturus palmatus* (Schneider) *Triton palmatus* Schneider PALMATE (WEBBED) NEWT	Amphibia Caudata	103, 104.
(154) *Triturus palmatus* (Schneider), see (153)		
(155) *Triturus viridescens* (Rafinesque), see (34)		
(156) *Urechis caupo* Fisher & MacGinitie INN KEEPER, HORSE COCK	Echiuroidea	3, 19, 29, 56, 61, 62, 65, 67, 77, 81, 82, 87, 89, 119.
(157) *Vespertilio noctula* Schreber, see (81)		
(158) *Vesperugo noctula* (Schreber), see (81)		
(159) *Xenopus laevis* Daudin CLAWED TOAD	Amphibia Caudata	86.

GENERAL INDEX

Except when there might be a misunderstanding, the word 'eggs' has been omitted, e.g. hexokinase means hexokinase in eggs. The word 'spermatozoa' has not been omitted in comparable cases.